2-Methoxyestradiol interferes with NFκB transcriptional activity in primitive neuroectodermal brain tumors: implications for management

Addanki P.Kumar, Gretchen E.Garcia, Jon Orsborn, Victor A. Levin and Thomas J.Slagen

Center for Cancer Causation and Prevention, AMC Cancer Research Center and University of Colorado Comprehensive Cancer Center, Denver, CO 80221, USA and 1Department of Neuro-Oncology, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Box 431, Houston, TX 77030, USA

Medulloblastoma (MB) is a primitive neuroectodermal tumor (PNET) of the central nervous system (CNS) and the most common malignant primary brain tumor in children. Currently, poor risk and recurrent MB patients are treated with cytotoxic chemotherapy alone or in combination with surgery and irradiation. In order to improve on therapeutic outcome and reduce toxicity of current treatment strategies, new and novel therapeutic agents are needed for MB patients. To that purpose, we have examined the effect of 2-methoxyestradiol (2-ME), an endogenous non-toxic estrogenic metabolite on the growth of three medulloblastoma cell lines (DAOY, D341 and D283); and two high-grade anaplastic astrocytoma/glioblastoma cell lines, U-87MG and T-98-G. We present evidence to show that 2-ME preferentially inhibits the growth of medulloblastoma cells significantly by blocking cell cycle progression predominantly in G2/M phase. 2-ME treatment results in phosphorylation of cdc25C without any significant alterations in the expression of cyclin B1 or p34cdc2. In addition, we observed a decrease in the levels of 14-3-3 proteins following treatment with 2-ME. Furthermore, 2-ME-mediated growth inhibition is accompanied by induction of apoptosis as evidenced by morphological alterations and DNA fragmentation analysis. Of interest is the finding that 2-ME induced apoptosis is not mediated through alterations in the expression of p53 or Bax and that transcriptional activity of NFκB and DNA binding activity is reduced indicating that 2-ME disrupts the NFκB signaling pathway. These results suggest that 2-ME may prove to be a useful therapeutic agent in the treatment of PNET brain tumors such as medulloblastoma. In addition, as 2-ME inhibits growth predominantly through G2/M block, it may enhance the effectiveness of radiation therapy.

Introduction

Medulloblastomas are uncommon primitive neuroectodermal tumors (PNET) of the cerebellum that accounts for 1.7% of primary central nervous system (CNS) tumors and are the most common malignant primary brain tumor of childhood (1). Medulloblastoma tumors are sensitive to a wide range of chemotherapeutic agents that include procarbazine, cisplatin and carboplatin, cyclophosphamide and ifosfamide, etoposide, methotrexate, lomustine, vincristine, dibromodulcite, melphalan and nitrogen mustard (2,3). Unfortunately, chemotherapy without radiation is not curative. There is, therefore, a continuing need for new chemotherapeutic agents as radiation therapy to the brain and spinal axis produces neurocognitive, hormonal, vertebral spine and cardiac toxicity (4–11).

2-Methoxyestradiol (2-ME) is a metabolic byproduct of estrogens, an anti-mitotic agent that is present in human urine and blood (12). 2-ME has been shown to inhibit the growth of various tumor cells through induction of apoptosis (13,14). 2-ME has also been shown to inhibit endothelial cell proliferation implicating its role in angiogenesis (15). To the best of our knowledge this is the only compound that has a dual advantage both as an anti-angiogenic and pro-apoptotic agent with an ultimate result of inhibition of tumor cell growth. Here we have investigated the effect of 2-ME on the growth of three medulloblastoma cell lines (DAOY, D341 and D283); two high-grade anaplastic astrocytoma/glioblastoma cell lines, U-87MG and T-98-G.

We report that 2-ME inhibits the growth of medulloblastoma cells significantly by predominantly blocking cells in G2/M phase of the cell cycle. Investigation of regulatory proteins involved in the G2/M-specific block following 2-ME treatment indicated phosphorylation of cdc25C without any significant alterations in the expression of Wee 1 kinase, cyclin B1 or p34cdc2. In addition, the levels of 14-3-3 proteins decreased following treatment with 2-ME. We also show that 2-ME activates caspase 3 leading to induction of apoptosis as revealed by phase-contrast microscopy and DNA laddering. In addition 2-ME induced apoptosis is not associated with any significant alterations in the expression of p53 or Bax, however, the transcriptional activity of NFκB promoter is reduced by 78%. Nuclear extracts prepared from 2-ME treated cells showed reduced NFκB DNA binding activity suggesting the potential deregulation of the NFκB signaling in mediating 2-ME’s anti proliferative activity. Furthermore, the dose required to inhibit the growth of these cells falls within therapeutically achievable levels indicating a prospective new agent for the treatment of tumors of CNS origin including medulloblastoma.

Materials and methods

Cell lines

Medulloblastoma cell line DAOY, high-grade anaplastic astrocytoma/glioblastoma cell lines U-87 and T-98-G were obtained from Dr Victor Levin (The University of Texas M. D. Anderson Cancer Center, Houston, TX). Cells were grown in Dulbecco’s Modified Eagle’s Medium containing high glucose, 10% fetal bovine serum and penicillin and streptomycin at 37°C with 5% CO2 (Life Technologies, Baltimore, MD). Additional medulloblastoma cell lines D283 and D341 were obtained from American Type Culture Collection (Rockville, MD) and grown according to the manufacturer’s recommendations.

Cell viability and proliferation assay

Actively growing cells (DAOY, D283, U-87MG and T-98-G) were plated in 96 well plates at a density of 4×103 cells/well in five replicates. After 24 h
in a 37°C incubator with 5% CO₂, the cells were treated with the indicated concentration of 2-ME. Control cells received only the vehicle (DMSO). Cell viability was determined by the trypan blue exclusion assay. Cell growth was monitored every 24 h using the CellTiter96 Aqueous one solution assay containing a tetrazolium compound (Promega Corporation, Madison, WI). This assay is based on the principle that actively growing cells generate reducing equivalents such as NADH that is necessary for the cells to reduce the tetrazolium compound to a formazan product. This was detected by measuring the absorbance at 570 nm using the Molecular Devices SpectraMaxPlus plate reader. An increase in the conversion of the MTS assay mixture contained 20 µl of cell lysate and 100 µl of firefly luciferase measuring buffer (LARI). Firefly luciferase activity was measured by Genios Tecan lumimeter (Phenix Research Products, Hayward, CA). After measuring the firefly luciferase activity, the reaction mixture was added to 100 µl of Renilla luciferase measuring buffer (Stop and Glow) and Renilla luciferase activity was measured. Renilla luciferase activity was used to normalize the transfection efficiency. Results are expressed as the ratio of firefly luciferase/ Renilla luciferase at equal amounts of protein. The data shown here are from a representative experiment that was performed four times with two different preparations of plasmid. For co-transfection experiments, the super repressor IxBox mutant expression vector (1 µg/well) was included along with the pNFkB reporter plasmid.

Electrophoretic mobility shift assays

Gel shift assays were done as described elsewhere (18). The double-stranded NFκB oligonucleotide was end labeled with [γ-32P]ATP using T4 polynucleotide kinase. Extracts were prepared as described above and incubated with the radiolabeled probe in binding buffer (containing 4 mM Tris–HCl, 12 mM HEPES, pH 7.9, 60 mM KCl, 0.5 mM EDTA, 1 mM DTT and 12% glycerol) for 25 min at room temperature in a final volume of 20 µl. After incubation, samples were fractionated on a 4% polyacrylamide gel in 0.25× TBE at 4°C. Following electrophoresis, the gel was dried and autoradiographed. For competition studies, the radiolabeled probe was mixed with 100-fold molar excess of unlabelled double-stranded synthetic NFκB oligonucleotide (homologous competition) and SPI (for heterologous competition) for 5 min prior to the addition of extracts.

Preparation of cell extracts and western blotting

Actively growing DAOY cells were treated with 3 µM 2-ME for 24 h. Following treatment, cells were lysed in a buffer containing [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.5% NP-40, 50 mM NaF, 1 mM NaVO₃, 1 mM phenylmethylsulfonyl fluoride, 25 µg/ml of leupeptin, 25 µg/ml of aprotinin, 25 µg/ml pepstatin and 1 mM DTT]. After passing the lysate through a 25 G needle, cell debris was removed by centrifugation at 12 000 r.p.m. for 30 min. Nuclear extracts were prepared according to the method of Dignam as described earlier (20). Protein content of the extracts was determined by the method of Bradford (17).

Equal amounts of cell extracts were fractionated on a 10% SDS–polyacrylamide gel (19). Following electrophoresis, proteins were electrophoretically transferred to a nitrocellulose membrane. The blotted membrane was blocked with 5% non-fat dried milk in Tris-buffered saline containing 0.1% Tween 20 (blocking solution), and incubated with indicated polyclonal antibodies (Santa Cruz Biotechnology, CA) followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG antibody (Sigma Chemical Co.) in blocking solution. Phospho-specific cdc25C antibody was obtained from Cell Signaling (Beverly, MA). Bound antibody was detected by enhanced chemiluminescence using Supersignal West Pico Chemiluminescent Substrate, following the manufacturer’s directions (Pierce, Rockford, IL).

Results and discussion

Inhibition of cell proliferation by 2-ME

We examined the effect of 2-ME on proliferation of medulloblastoma cells (DAOY and D283) and high-grade astrocytoma cells U-87MG and T-98-G using the CellTiter96 Aqueous one solution assay as described earlier (14). Exponentially growing cells were treated with 0, 0.5, 1, 2 and 3 µM of 2-ME and cell growth was measured every 24 h as described in the Materials and methods. As shown in Figure 1A and C, 2-ME inhibited the growth of DAOY and D283 medulloblastoma cells significantly. D341 cells grow largely as suspension cells unlike DAOY and D283 (that grow as attached and partially
2-ME induced apoptosis in brain tumors

Fig. 1. Effect of 2-ME on proliferation of medulloblastoma (DAOY, D283 and D341) and U-87 and T-98-G glioblastoma multiforme cells. DAOY cells were plated in 96 well plates as described in Materials and methods and treated with indicated concentrations of either 2-ME (A) or 16-epiestriol (B). Cell proliferation was measured by Cell Titer96 aqueous one solution assay at indicated time points by determining the absorbance at 570 nm using SpectraMaxPlus plate reader (Molecular Devices). The data shown here are an average ± SD of five replicate wells and are representative of four independent experiments. D283 cells were plated and treated as above (C). However, cell proliferation was measured following 72 h of treatment with indicated concentrations of 2-ME. The effect of 2-ME on D341 cells (that grow as suspension cells) was determined by colony formation assay (D). Cells were plated in triplicate in 35 mm dishes on 0.5% agarose containing media as described in Materials and methods. Following 14 day incubation, cells were stained with 0.5 ml of 0.02% p-iodonitrotetrazolium and colonies were counted in 10 different fields from each plate. The results were expressed as mean values ± SD. DAOY cells were used as positive control and wells containing no cells were used as negative control. The data shown here are an average ± SD of five replicate wells and are representative of four independent experiments. Effect of 2-ME on T-98-G and U-87 was essentially determined as described above for DAOY cells (E and F, respectively). Based on these data, the IC50 and IC10 values (concentration of 2-ME required to show 50% and 10% of growth inhibition) following incubation with 2-ME for 72 h were determined as shown in Table I.

Table I. The activity (IC50 and IC10) of 2-ME on cells grown in culture

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC50 (µM)</th>
<th>IC10 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAOY</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>U87-MG</td>
<td>3</td>
<td>&gt;50</td>
</tr>
<tr>
<td>T-98G</td>
<td>6</td>
<td>50</td>
</tr>
</tbody>
</table>

Attached, respectively), therefore we could not measure the effect of 2-ME on D341 cells using the cell proliferation assay. Hence, we determined the effect of 2-ME on anchorage-independent growth of D341 cells on soft agar. As a positive control and also to confirm the results from the above cell proliferation-based assay, we used DAOY cells. As shown in Figure 1D, anchorage-independent growth of DAOY and D341 cells on soft agar was significantly inhibited by 2ME that is consistent with the above cell proliferation data from DAOY cells (Figure 1A). In contrast, the control cells or the cells treated with 16-epiestriol continued to proliferate during the course of the experiment (Figure 1B). The data indicate that 2-ME is a potent inhibitor of proliferation of medulloblastoma cells and the observed inhibitory effect is specific to 2-ME. As 16-epiestriol is an inactive analog of 2-ME that lacks the methoxy group at the second position, these results also indicate the importance of the methoxy moiety for the observed growth inhibitory potential of 2-ME consistent with previous reports from our laboratory and other studies (14). These results were also confirmed by measuring cell viability using the trypan blue exclusion method (data not shown).

In order to determine the specificity of 2-ME towards tumor cells, we compared the effect of 2-ME on DAOY cells with non-tumorigenic T-98-G anaplastic astrocytoma cells. As shown in Figure 1E and Table I, 2 µM 2-ME caused a 38% decrease in the growth of T-98-G cells compared with 80% for DAOY cells in 72 h (Figure 1A). In order to produce comparable effects, a concentration of >10 µM was necessary. These results indicate that 2-ME works very efficiently in tumor cells compared with non-tumorigenic astrocytoma cells. The data are also consistent with published data showing the specificity of 2-ME in inhibiting the growth of tumor cells (12–15).
Fig. 3. 2-ME inhibits the growth of DAOY cells through alterations in cell cycle distribution. DAOY cells were treated with either DMSO alone or with 3 µM of 2-ME for 24 h as described in Materials and methods. Following treatment, cells were harvested washed with PBS and then resuspended in 1 ml of Krishan stain containing 1.1 mg/ml of sodium citrate; 46 µg/ml of propidium iodide; 0.01% of NP-40 and 10 µg/ml of RNase (A). For quantification of apoptotic cells, cells were resuspended in a stain containing 0.3% Saponin and 25 µg / ml of PI (B). Data were analyzed using Modfit LT and the numbers of apoptotic cells are shown as percent apoptotic cells in (B). Alterations in the distribution of cells in different phases is shown as a graph in (C). Number of condensed chromosomes was measured following treatment with different concentrations of 2-ME (0.5, 1, 2, 3 and 5 µM for 24 and 48 h) as described in Materials and methods. Cells were examined from 10 different fields of view and mitotic indices were calculated as the number of cells with condensed chromosomes (D). The percent condensed chromosomes was calculated by setting the number obtained with untreated cells at 100. The data shown here are an average ± SD of two independent experiments.

Morphological characteristics of DAOY cells following 2-ME treatment

Studies to investigate the mechanism of 2-ME induced growth inhibitory activity were conducted in DAOY cells. Exponentially growing DAOY cells at 70–80% confluence were treated with 2-ME (0.5 and 1 µM). The cells were observed every 24 h for morphological changes associated with 2-ME treatment. As shown in the Figure 2, following treatment, significant morphological alterations including rounding of cells, shrinkage of cells, retraction from their neighboring cells and condensation of the cytoplasm were observed. A significant proportion of the cells started to float by 24 h and finally detached from the dishes. Under identical conditions, the vehicle or 16-epiestriol did not induce any features of apoptosis.

Cell cycle distribution of DAOY cells following 2-ME treatment

In order to determine the mechanism of growth inhibition by 2-ME, we examined the effect of 2-ME on cell cycle distribution by FACS analysis. The representative flow cytometric histogram shown in Figure 3A indicates DAOY cells treated with 2-ME (3 µM) for 24 h showed approximately a 2–4-fold increase in the G2/M population with a concomitant decrease in population of cells in G1 and S phase. Staining of cells with Saponin showed that ~9–20% of the cells were undergoing apoptosis (Figure 3B). Figure 3C is a graphical representation (average of two independent experiments) of cells in each phase following treatment with 2-ME for 24 h. In addition, we have also observed a subG1 peak (indicated by an arrow) suggestive of the presence of apoptotic cells with fragmented DNA (20). However, treatment of cells with 16-epiestriol had no significant effect either on the distribution of cells in the cell cycle or on apoptosis. The data indicate that 2-ME inhibits growth of DAOY cells by arresting the cells predominantly in the G2/M phase and induces apoptosis.

To investigate whether the 2-ME induced accumulation of cells in G2/M phase was due to a specific block in G2 or in M phase, we measured the mitotic index of DAOY cells following treatment with 2-ME as described earlier (14). As shown in Figure 3D, the number of condensed chromosomes decreased with increasing concentrations of 2-ME compared with untreated cells. DAOY cells treated with 0.5 µM of 2-ME for 24 h showed a decrease from 100 to 40 (60% decrease) that was further decreased to 15% at 1 µM concentration. The
data indicate that 2-ME treatment blocks cells in G₂ resulting in failure of cells to enter mitosis.

**Induction of apoptosis in DAOY cells following 2-ME treatment**

As the cell cycle data along with morphological alterations of cells following 2-ME treatment indicated induction of apoptosis as a potential mechanism for 2-MEs antiproliferative activity, we sought to gather evidence for induction of apoptosis in these cells. Apoptosis is an active genetically controlled process by which cells self-destruct and is accompanied by characteristic morphological and biochemical changes (21). One of the earliest events during this process is the activation of a calcium-dependent endonuclease associated with nuclear DNA fragmentation (21). During this process chromosomal DNA is degraded primarily into large DNA fragments followed by subsequent formation of smaller oligonucleosomal fragments resulting in the appearance of a defined DNA ladder when analyzed by agarose gel electrophoresis. Genomic DNA from control and 2-ME treated DAOY cells were resolved by electrophoresis. As shown in Figure 4A, DNA fragmentation resulting from internucleosomal cleavage was clearly visible in DAOY cells treated with 2-ME for 24 h. In contrast, we did not detect any evidence of fragmentation in the control cells. The data show that 2-ME causes DNA fragmentation that is characteristic of apoptosis.

Further, we confirmed the induction of apoptosis by measuring caspase activity. Activation of a series of cytosolic proteases called caspases has been shown to be involved in the induction of apoptosis by a wide variety of agents and extracellular signals resulting in the cleavage of various cellular protein substrates leading to impairment of tissue homeostasis with ultimate destruction of the cell (22,23). Among these, caspase 3 has been implicated as a key protease that is activated during the early stages of apoptosis and is detected only in cells undergoing apoptosis. As shown in Figure 4B no caspase activity was detected in the extracts prepared from untreated or 16-epiestriol treated cells. In contrast, caspase 3 activity was detected in extracts prepared from cells treated with 3 µM 2-ME. This 2-ME induced caspase activity was reduced by ~64% when extracts were prepared from cells treated with Z-VAD-FMK, a caspase inhibitor along with 2-ME, indicating the involvement of caspase 3 in this process. Taken together, these results demonstrate that 2-ME inhibits the growth of DAOY cells through induction of apoptosis.

**Mechanism of 2-ME induced apoptosis in DAOY cells**

The tumor suppressor protein p53 is a key regulator of cell cycle checkpoint and can induce apoptosis in a wide variety of experimental systems through distinct pathways (24–27). It is also well established that the tumor suppressor function of p53 is mediated by accumulation of wild-type p53 in response to extracellular signals with sequential induction of either cell cycle arrest or apoptosis. Mutation of p53, which is very frequent in human cancers (50%), is the result of the disruption of these signaling pathways. Deregulation of such signaling pathways ultimately provides a selective growth advantage to tumor cells. In addition, p53 mutations provide a basis for better radiation and chemotherapy sensitivity and, therefore, a longer tumor response to treatment. This is seen, for instance in patients with anaplastic astrocytoma. In order to test whether 2-ME induced apoptosis was mediated by alterations in the levels of p53, and the apoptosis proteins that it regulates such as Bcl-2 and Bax, the level of these proteins was examined in DAOY cells by western blot analysis. As shown in Figure 5 there was no significant change in the levels of p53 or...
Bax indicating that the observed apoptosis following 2-ME treatment may not involve p53 or Bax. 2-ME is known to induce apoptosis both through p53-dependent and p53-independent fashion in human cancer cell lines depending on the tissue and cell type (13, 14, 28).

Effect of 2-ME on the expression of G2/M-specific proteins

It is believed that the initiation of apoptosis can take place at any phase in the cell cycle. The flow cytometric data demonstrated that 2-ME arrested the growth of cells at the G2/M phase. Progression through cell cycle is regulated by sequential activation and subsequent inactivation of a series of cyclin-dependent kinases (cdks). The activities of Cdks are regulated positively by cyclins and negatively by cdk inhibitory proteins in response to a wide variety of anti-proliferative signals. As 2-ME inhibits the growth of medulloblastoma cells by predominantly blocking the cells in G2/M phase of the cell cycle, we investigated the alterations in the critical regulatory proteins known to participate in this phase of the cell cycle by immunoblot analysis (29). Activation of the p34cdc2 kinase complex is known to modulate the progression of cells from G2 in to the M phase of the cell cycle by promoting chromosome condensation, cytoskeletal reorganization and nuclear envelope breakdown. The kinase activity of cdc2 is controlled during the cell cycle both by the association with cyclin B1 and by phosphorylation/dephosphorylation on the inhibitory phosphorylation sites, Thr-14 and Tyr-15. The cyclinB/cdc2 complex is inactivated as long as cdc2 15-tyrosine/14-threonine is phosphorylated (mediated by Wee 1, Myt 1 and related kinases) and dephosphorylation of these residues by cdc25 phosphatases enables cdc2 activation and entry into mitosis. In mammalian cells, cdc25 phosphatases are encoded by a multigene family consisting of cdc25A, cdc25B and cdc25C that control distinct aspects of cell cycle progression. Cdc25A is vital for entry into S phase while Cdc25B is essential for pre-initiating G2/M transition and S phase progression; Cdc25 C activates the cdc2/cyclinB complex through a dephosphorylation reaction on threonine 14 and tyrosine 15 that is required for entry into mitosis (29, 30). Furthermore, association of phosphorylated cdc25 C with 14-3-3 proteins prevents dephosphorylation of cdc2 that is critical for G2/M progression. Thus, the observed G2/M block in medulloblastoma cells by 2-ME may be due to alterations in the levels and activity of Wee1 kinase, cdc25 C and/or cdc2. We investigated whether 2-ME induces G2/M arrest by alterations in the protein levels of Wee1 kinase, p34cdc2, Cyclin B1, cdc25B and cdc25 C following treatment with 2-ME (3 μM for 24 h). All blots were stripped and probed with β-actin in order to check for equal loading of the protein samples.

Western blot analysis indicates no significant changes in the steady-state levels of p34cdc2, cyclinB1 or cdc25B (Figure 5). Wee1 kinase was not detected either in extracts from untreated as well as 2-ME treated cells. This is consistent with our previous data showing no expression of Wee 1 kinase in extracts from control prostate cancer LNCaP cells (14). However, in these prostate cancer cells, Wee 1 became detectable following 2-ME treatment unlike DAOY cells. This could be a cell type-specific effect or DAOY cells may utilize another pathway in mediating 2-MEs antiproliferative activity. Expression of cdc25 C showed a clear shift in the mobility of the bands following treatment with 2-ME. The observed altered mobility may be due to differential phosphorylation of cdc25 C. With this background, we examined whether 2-ME treatment leads to phosphorylation of cdc25 C using an antibody that specifically detects phosphorylated cdc25 C. As shown in Figure 6, we did not detect any phosphorylated cdc25 C in the control extracts; however, following treatment with 2-ME the phosphorylated band was detectable. In addition, the level of 14-3-3 proteins in extracts from 2-ME treated cells became undetectable using 14-3-3 β antibody that is broadly reactive among its family members. Although we do not know the exact role of cdc25C in 2-ME-mediated G2/M block, based on our observation we speculate that 2-ME treatment possibly leads to phosphorylation of cdc25C, which in turn associates, with 14-3-3 proteins. This could lead to hyper-phosphorylation of cdc2; making it inactive and blocking cells in G2/M (31).

For co-transfection experiments, the super repressor IκBα mutant expression vector (1 μg/well) was included along with the pNFκB reporter plasmid. (B) Electrophoretic mobility shift assay (EMSA) of nuclear extracts prepared from control or 2-ME treated cells using NFκB consensus oligonucleotide as radiolabeled probe. Nuclear extract (5 μg) was incubated with ~0.2 ng of labeled probe as described in the Materials and methods and the DNA-protein complexes were resolved on a 4% non-denaturing gel by electrophoresis. Unbound and bound complexes (I, II and III) are indicated by arrows.

Role of transcription factor NFκB in 2-ME induced antiproliferative activity

The transcription factor NFκB has been shown to play an important role in coordinating the control of apoptotic cell death, which either promotes or inhibits apoptosis depending on the cell type and the apoptotic stimuli (33). Activation of NFκB has been shown to be associated with decreased levels of apoptosis in vitro and mice lacking p65 exhibit increased levels of apoptosis (34, 35). We have hypothesized that 2-ME may induce apoptosis through inhibition of NFκB-mediated apoptosis.
cell survival signaling pathway in DAOY cells. We have examined the contribution of NFκB in mediating 2-ME induced apoptosis using transient transfection expression assays using NFκB-luciferase reporter vector containing four tandem copies of NFκB consensus sequence (Clontech Laboratories) and NFκB DNA binding activity in nuclear extracts prepared from cells treated with 2-ME.

As shown in Figure 6A, the NFκB promoter was constitutively activated in DAOY cells and following treatment with 2-ME (3 μM for 2 h), the transcriptional activity of the NFκB promoter was reduced by 78%. In addition, co-transfections performed with mutant IκBα super repressor (containing mutated serine residues at 32 and 36) blocked this constitutive activation of NFκB promoter activity that is consistent with previous reports (36). The data indicate that NFκB/IκBα signaling has a role in mediating 2-MEs antiproliferative activities observed in DAOY cells. The detailed mechanism whether this is through inhibition of IκBα phosphorylation and degradation of IκBα is under investigation.

We have also tested the ability of nuclear extracts prepared from DAOY cells treated with 2-ME to bind the NFκB oligonucleotide in electrophoretic mobility shift assays (EMSA). As shown in Figure 6B, extracts prepared from untreated cells showed one major (I) and two minor complexes (II and III). All three DNA–protein complexes were abolished with an excess (100-fold molar) of unlabeled consensus NFκB but not with Sp1 oligonucleotide indicating the specificity of the interaction (data not shown). Extracts prepared from 2-ME treated cells did not show the DNA–protein complex formation. This correlates with the observed decrease in the transcriptional activity of the NFκB promoter following 2-ME treatment. The data indicate that interference with NFκB signaling pathway with 2-ME could contribute to the observed antiproliferative activity with consequent induction of apoptosis.

Conclusions

Current treatment strategies for patients with medulloblastoma are a function of patient stratification into two major risk groups (37,38). The ‘average risk’ group includes patients with localized disease of the cerebellum and total or near-total surgical resections. Approximately 60–70% of patients fall into this category and have ~60% chance of 5-year progression-free survival after treatment with craniospinal irradiation. Conversely, patients with disseminated disease or partially resected tumor constitute a ‘poor-risk’ group with an ~40% likelihood of 5-year progression-free survival after treatment with craniospinal irradiation and possible adjuvant chemotherapy. While medulloblastoma tumors are sensitive to a wide range of chemotherapeutic agents, chemotherapy without radiation therapy is rarely curative. There remains, therefore, a continuing need for new and novel chemotherapy agents since radiation therapy to the brain and spinal axis produces substantial long-term morbidity.

Much pre-clinical work would need to be done before concluding the 2-ME should move forward into clinical trials for PNET; however, we believe that if efficacy can be validated in several rodent medulloblastoma models it will be found that 2-ME is well tolerated with little systemic toxicity. It is presently unclear what effect 2-ME has on the highly proliferating pool of non-neoplastic cells in the pediatric age group. Under usual physiological conditions, plasma 2-ME is in the picomolar range, but increases during pregnancy (39,40). In various pre-clinical studies it has been shown that the plasma levels of 2-ME attained at efficacious doses are similar to the levels attained normally during the third trimester of pregnancy (40) indicating that therapeutically effective doses of 2-ME would not be expected to be harmful even to a developing fetus at late stages of pregnancy. In addition, the biological activity of 2-ME appears to be tumor specific, since very little or no toxicity has been observed in normal tissues or in in vivo experiments (41). For example, 2-ME has been shown to induce apoptosis in transformed breast epithelial cells (MCF-7) (42), lung carcinoma cells (43), transformed skin fibroblasts cells, but not in normal fibroblasts (44). 2-ME also had no effect on either cell viability or apoptosis of normal bronchial cells (45). Based on these observations, even if 2-ME is found to have an effect on non-neoplastic cells, it is unlikely to be a toxic effect.

Our results suggest that 2-ME should be evaluated further in pre-clinical studies as a potential agent for the treatment of medulloblastoma and possibly other PNET brain tumors. Use of 2-ME has numerous advantages: (i) 2-ME is a natural endogenous estrogen metabolite that reaches micromolar levels during pregnancy (12); (ii) 2-ME is also active when given orally and is relatively non-toxic (12); (iii) 2-ME has been shown to inhibit cell proliferation and also formation of new blood vessels necessary for tumor cell proliferation (15). Although toxicity of normal neurons and glia to 2-ME is not known, the differential sensitivity of 2-ME towards different cell lines in our study suggests that it may have little or no effect on these cells. Along these lines it has been shown that 2-ME specifically targets tumor cells without affecting non-proliferating cells indicating that many normal tissues may be relatively resistant to the toxic effects of 2-ME (as discussed above and ref. 15). Unraveling the mechanisms of 2-ME induced apoptosis may lead to more effective medical therapy for PNET tumors with less long-term morbidity and new opportunities for drug and radiation combinations.

Acknowledgements

We thank our colleagues at AMC Drs Rita Ghosh, Robert Strange and Jaime Modiano for many suggestions and stimulating discussions; Dr Henry Thompson for use of Plate reader. We acknowledge the University of Colorado Comprehensive Cancer Center Flow Cytometry Core facility for assistance with flow cytometric analysis. This work was supported in part by funds from Oncology Sciences Corporation Inc. and Cancer League of Colorado (A.P.K.). J.O. was a summer student supported by research grants from NCI Cancer Education Grant R25 CA49981 and ACS Colorado Division, Brooks Trust. We acknowledge receiving the expression plasmid IκBα (S32/36) mutant from Dr Z.-G.Liu (National Institute of Health, Bethesda, MD).

References


