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2-METHOXYESTRADIOL INDUCES CASPASE-INDEPENDENT, MITOCHONDRIA-CENTERED APOPTOSIS IN DS-SARCOMA CELLS

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The anti-cancer potential of the natural estrogen metabolite 2-methoxyestradiol is associated with microtubuli interaction, anti-angiogenetic effects and inhibition of superoxide dismutase leading to apoptosis. The effectors of apoptotic signaling through 2-methoxyestradiol, however, are cell type-dependent. We investigated the effect of 2-methoxyestradiol on several events associated with apoptosis in rat DS-sarcoma cells. Translocation of the pro-apoptotic protein Bax to mitochondria was identified as an initial apoptotic event that was accompanied by a decrease in mitochondrial transmembrane potential and the formation of reactive oxygen species (ROS) followed by mitochondrial release of apoptosis inducing factor and endonuclease G. In addition, 2-methoxyestradiol treatment caused upregulation of death receptor ligands FasL and TNFα and induced caspase-8 activation. The pan caspase inhibitor Z-VAD-FMK did not suppress apoptotic cell death, however, indicating that the major pro-apoptotic effect of 2-methoxyestradiol is mediated by a caspase-independent mechanism. Furthermore, ROS do not seem to play a pivotal role in the toxic/apoptotic effect of 2-methoxyestradiol in DS-sarcoma cells because supplementation with various antioxidants provided only limit protection. Colony formation was not affected by antioxidants. Therefore, in DS-sarcoma cells, the breakdown of mitochondrial integrity with the subsequent release of mitochondrial nucleases is the main factor in 2-methoxyestradiol mediated cell death.

Key words: *2-methoxyestradiol; apoptosis; Bax; AIF; endonuclease G; mitochondria; reactive oxygen species*

The estrogen metabolite 2-methoxyestradiol (2-ME) is a highly potent anti-cancer agent that effectively induces apoptosis in tumor cells, both *in vitro* and *in vivo*.[1] Although 2-ME is formed from 17β-estradiol,[2] its anti-cancer action is not dependent on estrogen receptor binding[3] and several molecular mechanisms have been attributed to 2-ME-mediated cell death and apoptosis. 2-ME has a strong anti-angiogenic effect and interacts with microtubuli, resulting in cell cycle arrest at G2/M phase.[4] D'Amato *et al.*[5] reported that 2-ME inhibits tubulin polymerization by interacting with the colchicine site. 2-ME also increases the insoluble polymerized fraction of cellular tubulin similar to the anti-cancer drug Taxol.[6]

In addition, 2-ME increases the superoxide anions, by inhibiting superoxide dismutase (SOD).[7] Investigations of different estradiols and estrones showed that an alkoxy-group at position 2 is responsible for the SOD-inhibiting activity.[8] In leukaemia cells, the 2-ME-mediated inhibition of SOD seems to be of great importance because in these cells ROS-formation is crucial for apoptosis induction that has been shown by ambroxol, a superoxide anion quencher and the antioxidant N-acetyl-cysteine inhibit 2-ME-induced apoptosis.[7] This mechanism may not apply to all cell lines: in hepatoma cells, 2-ME treatment also leads to both generation of reactive species and apoptotic cell death, however, the antioxidant magnolol did not inhibit apoptosis despite of its ability to reduce ROS-formation.[9]

Irrespective of the role of ROS, mitochondria are likely to represent the major site involved in 2-ME-induced apoptosis because 2-ME induces events like translocation of Bax to the mitochondria,[8] decrease in mitochondrial transmembrane potential,[7] release of cytochrome c[7,8] and activation of effector caspases.[9] Phosphorylation of Bcl-2 and Bcl-x_L was found in 2-ME exposed pancreatic cancer and leukaemia cells,[6,8] but the functional consequences of this post-translational modification remain controversial. Several reports suggested that phosphorylation of Bcl-2 and Bcl-x_L leads to inactivation of its anti-apoptotic function. Ling and Fan,[10,11] however, demonstrated that Bcl-2 phosphorylation is a marker of mitotic arrest rather than a determinant of apoptosis.

There is evidence for additional targets of 2-ME mediated apoptosis that point to the involvement of death receptor signaling. LaVallee *et al.*[12] demonstrated that 2-ME upregulates death receptor 5 (DR5) in the human breast carcinoma cell line MDA-MB-231 *in vitro* and *in vivo*. In endothelial cells, 2-ME treatment increases expression of another death receptor, CD95.[13]

Our study was aimed at understanding the contribution of caspases, mitochondria and death receptor signaling in the apoptotic process of 2-ME treated sarcoma cells. Furthermore, the formation of reactive oxygen species (ROS) was investigated to address the question of whether they represent the initial trigger event for the apoptotic process or are the consequence of mitochondrial breakdown.

MATERIAL AND METHODS

Material

All the chemicals were obtained from Sigma (Deisenhofen, Germany) unless otherwise indicated. Cell culture materials were purchased from Biochrom (Berlin, Germany) or Greiner (Frickenhofen, Germany).

Cell culture

DS-sarcoma cells of the rat were used for *in vitro* experiments. Cells were grown in RPMI medium supplemented with 10% FBS and 2 mM glutamine at 37°C in a humidified 5% CO_2 atmosphere. They were passaged twice a week.

Drug treatment

2-ME was dissolved in absolute ethanol to give a 20 mM solution and stored at −20°C. Cells were treated with 2-ME for up to 96 hr. The concentration of ethanol in the medium of 2-ME treated and control cells was adjusted to 0.1 % (v/v). As demonstrated in a previous study[14] saturating effects of 2-ME only

Abbreviations: AIF, apoptosis inducing factor; COX, cytochrome oxidase; DR5, death receptor 5; endoG, endonuclease G; PARP, poly (ADP-ribose) polymerase; PTP, permeability transition pore; ROS, reactive oxygen species; 2-ME, 2-methoxyestradiol; SOD, superoxide dismutase; TNFα, tumor necrosis factor alpha; VDAC, voltage dependent anion channel.

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occurred at concentrations >5 μM. In our study, concentrations >5 μM 2-ME were not used.

Determination of ROS

ROS production was assessed by oxidation of 2′,7′-dichlorodihydrofluorescein diacetate (H_2-DCF-DA) (Molecular Probes, Leiden, The Netherlands) to the fluorescent product 2′,7′-dichlorofluorescein (DCF). In the presence of ROS (especially hydrogen peroxide and lipid hydroperoxides), H_2-DCF is rapidly oxidized to highly fluorescent DCF.[15] Cells were stained as described previously.[16] 10^6 cells were incubated in RPMI with 5 μM H_2-DCF-DA for 45 min at 37°C, then incubated in PBS containing 0.2 μg propidium iodide/ml for 10 min at room temperature. Thereafter, cells were washed once and resuspended in 1 ml PBS. Flow cytometric analysis was carried out within 1 hr (525/620 nm).

Detection of mitochondrial changes

During apoptosis, mitochondrial permeability is altered. Specifically, the discontinuity of the outer mitochondrial membrane results in the redistribution of cytochrome c to the cytosol followed by depolarization of the inner mitochondrial membrane. Binding of cytochrome c to Apaf-1 promotes activation of caspase-9 and subsequent activation of downstream-caspases such as caspase-3. The mitochondrial membrane potential ($\Delta\Psi_m$) was determined using rhodamine 123 as described by Li *et al.*[17] Treated cells were washed and resuspended in medium at 10^6 cells/ml. After incubation with 5 μM rhodamine 123 for 20 min at room temperature, cells were washed once in medium and resuspended in PBS for flow cytometry analysis (575 nm). Carbonyl cyanide m-chlorophenylhydrazone (CCCP), added at the concentration of 50 μM, was used as positive control.

Swelling of mitochondria is another mark of apoptosis.[18] Relative mitochondrial mass was measured by flow cytometry using 10-nonyl-acridine-orange (NAO) (Molecular Probes, Leiden, The Netherlands). The fluorescent dye NAO binds specifically to the mitochondrial inner membrane, independent of energetic state.[19]

Cells (10^6) were washed once in PBS and stained with 10 μM NAO in 1 ml PBS for 10 min at room temperature. Thereafter cells were washed in PBS and underwent flow cytometry analysis (525 nm).

Caspase-3 and -8 activity assay

Caspase-3 activity was determined as described previously.[14] The activation of caspase-8, an intracellular cysteine protease, plays an important role in death receptor mediated apoptosis. Once activated, caspase-8 cleaves Bid or activates effector caspases such as caspase-3.

After drug treatment, cells were washed in PBS and 4×10^6 cells were lysed in 300 μl ice cold HEPES buffer (10 mM HEPES, pH 7.4, 2 mM EDTA, 1 g/l CHAPS, 2 mM Pefabloc [Biomol, Hamburg, Germany]). The solution was centrifuged (20,000*g*) at 4°C for 30 min and 200 μl of the supernatant was mixed with 22 μl 50 mM dithiothreitol and stored at −80°C until the caspase-8 activity assay was carried out.[20] The rest of the supernatant was used for protein content determination (Bio-Rad DC Protein Assay). For caspase-8 activity measurement, 100 μl of the supernatant was incubated with the fluorogenic caspase-8 tetrapeptide substrate Ac-IETD-amino-4-methylcoumarin (Bachem, Heidelberg, Germany) at a final concentration of 50 μM.[21] Cleavage of the substrate was followed by determination of emission at 460 ± 40 nm after excitation at 360 ± 40 nm using the fluorescence plate reader FL600 (Biotek, Winooski, VT). Relative caspase-8 cleavage activity was expressed as a function of protein content.

DNA fragmentation

A late biochemical hallmark of apoptosis is the fragmentation of the genomic DNA. It is an irreversible event and occurs before changes in plasma membrane permeability.

Cells (1.5×10^6) were resuspended in 500 μl lysis buffer (10 mM Tris, pH 8.0, 100 mM NaCl, 25 mM EDTA, 5 g/l SDS). The solution was incubated sequentially with 50 μg/ml RNase A (Qiagen, Hilden, Germany) for 60 min at room temperature and 100 μg/ml proteinase K (Qiagen, Hilden, Germany) at 50°C overnight. Subsequently, the solution was cooled to room temperature and gently extracted with 500 μl Roti Phenol/Chloroform (Roth, Karlsruhe, Germany). The mixture was transferred to a Phase Lock Gel light tube (Eppendorf, Hamburg, Germany). After centrifugation at 14,000*g* for 5 min the upper aqueous phase was decanted and 0.1 vol 3 M sodium acetate and 2.2 vol of absolute ethanol added. The tube was kept at −20°C for 2 hr. After centrifugation, the DNA precipitate was washed with 70% ethanol and dried for 20 min at room temperature. DNA was dissolved in TE buffer (pH 8.0) overnight at 4°C. To detect DNA fragments, 2 μg of isolated DNA was separated on a 1.8% agarose gel in TBE buffer and stained with ethidium bromide. DNA fragments were visualized under ultraviolet light.

Real time RT-PCR

mRNA expression of FasL and TNFα, 2 ligands of death receptors (Fas and TNF-R1) were measured quantitatively by real-time RT-PCR.

Total RNA isolation was done with RNeasy mini kit (Qiagen, Hilden, Germany). Two micrograms of total RNA were reverse transcribed into cDNA using Omniscript RT Kit (Qiagen) and oligo dT-primers (end volume = 20 μl) according to manufacturer's instructions. The real-time reactions were carried out in an iCycler iQ (Bio-Rad, Munich, Germany) using a QuantiTect SYBR Green PCR kit (Qiagen). The reaction mixture was prepared to contain 1× QuantiTect Mix, 1 μl fluorescein (0.5 μM; Bio-Rad), 1 μM of each primer, 1 μl cDNA and Rnase-free water to 50 μl.

The following primers were used for RT-PCR: GAPDH, 5′-GTG TTC CTA CCC CCA ATG TAT-3′ and 5′-CCTGTTGCT GTA GCC ATA TTC-3′; TNFα, 5′-CAG ATG GGC TGT ACC TTA TC-3′ and 5′-GGA CTC CGT GAT GTC TTA GTA-3′; and FasL, 5′-TCT GGA ATG GGA AGA CAC ATA-3′ and 5′-ACC AGA TCC CCA GGA TAC TT-3′.

Fluorescein served as passive reference dye to correct well-to-well variations. A calibration curve was run in parallel and in duplicate with each analysis, using PCR fragments of the target cDNA in a concentration of 10^1–10^9 copies per sample. No template controls (ddH_20) were included in every analysis. The PCR program was as follows: 95°C for 15 min, 40 cycles at 95°C for 30 sec, 57°C for 30 sec (61°C for TNFα), 72°C for 30 sec. The fluorescence values were collected at 72°C. At the end of the PCR, a melt curve analysis was carried out by gradually increasing the temperature to 95°C to confirm the specificity of the chosen primers as well as show the presence of primer-dimers. The post run data were analyzed with iCycler iQ real time detection system software version 3.0 (Bio-Rad, Munich, Germany) to transform the SYBR Green fluorescent signal into a relative number of copies of target molecules. Differences in cDNA amount were equalized by expression of the house-keeping gene GAPDH.

Western blot analysis

DS-sarcoma cells (20×10^6) were washed after the indicated treatment periods. The cells were resuspended in 1 ml of a hypotonic buffer (20 mM HEPES-KOH [pH 7.5], 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 5 μl protease inhibitor cocktail/ml, 250 mM sucrose), and incubated for 20 min on ice. They were then homogenized in a tissue grinder. The homogenate was centrifuged at 100*g* for 10 min at 4°C, and the resulting supernatant was then centrifuged at 10,000*g* for 30 min at 4°C. The second pellet containing mitochondria was suspended in 40 μl lysis buffer (10 mM Tris, 9 g/l NP-40, 1 g/l SDS, 5 μl/ml protease inhibitor cocktail, pH 7.5). The supernatant contained the cytosolic cell fraction.[22] Isolation of nucleoli for endoG determination was carried out as described by Staal *et al.*[23] The amount of protein was measured by Bio-Rad DC protein assay.

Equal protein amounts were run on a 10 or 12% polyacrylamide gel and blotted onto PVDF membranes by semidry electroblotting. Membranes were stained with Ponceau S to verify equal protein loading per lane.

Western blot detection

After blocking (3% milk powder and 0.05% Tween 20 in TBS) the membranes for 1 hr, blots were probed for 1 hr with primary antibody and rinsed twice with TBST. The membranes then were probed with horseradish peroxidase-conjugated secondary antibody for 1 hr. After 3 washes in TBST the detection was carried out with chemiluminescence system LumiGLO (Cell Signaling Technology, Beverly, MA).

Following antibodies were used for Western blot detection: goat polyclonal anti-AIF antibody, mouse monoclonal anti-Bax antibody, mouse monoclonal anti-Bcl-2 antibody, mouse monoclonal anti-Bcl-x_L antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) and mouse monoclonal anti-cytochrome oxidase (subunit IV) antibody (Molecular Probes, Leiden, The Netherlands). Rabbit polyclonal anti-endoG antibody was kindly provided by Dr. Wang, University of Texas Southwestern Medical Center, Dallas. As secondary antibody anti-mouse IgG peroxidase conjugate (Calbiochem, La Jolla, CA) and anti-rabbit IgG horseradish peroxidase linked (Cell Signaling Technology) were used. As internal controls following antibodies were used: cytosolic fraction: rabbit polyclonal anti-actin antibody (Santa Cruz Biotechnologies); mitochondrial fraction: mouse monoclonal anti-cytochrome oxidase subunit IV (COX) antibody (Molecular Probes); nuclear fraction: rabbit polyclonal anti-histone H2B antibody (Cell Signaling Technology).

Statistical analysis

Results are expressed as means ± SEM. Differences between the groups were assessed by two-tailed Wilcoxon test for unpaired samples. The significance level was set at $\alpha = 5\%$ for all comparisons.

RESULTS

ROS-generation and mitochondrial membrane potential

To investigate ROS-formation after 2-ME treatment in DS-sarcoma cells, we determined oxidation of H_2-DCF by flow cytometry. As shown in Figure 1*a*, ROS-formation occurred after 1.5 hr of 2-ME treatment and increased during the first 12 hr. In comparison to control cells, a significant increase in ROS formation was observed after 6 hr and 12 hr for both concentrations ($p < 0.05$). Cells treated with 5 μM 2-ME had higher ROS-levels than those treated with 1 μM 2-ME, but this difference was statistically not significant.

An inverse relationship was observed for the mitochondrial transmembrane potential ($\Delta\Psi_m$) in experiments where DS-sarcoma cells were stained with rhodamine 123, which binds to the mitochondrial transmembrane dependent on its potential. In addition to the potential, rhodamine 123 fluorescence is also dependent on the amount of mitochondrial membrane,[24] thus rhodamine 123 fluorescence was corrected for NAO-fluorescence, an indicator for mitochondrial mass. As shown in Figure 1*b*, $\Delta\Psi_m$ of DS-sarcoma cells decreased immediately after 2-ME administration, although the difference between 1 and 5 μM 2-ME was not as large as for ROS formation (Fig. 1*a*). In addition, the magnitude of ROS-formation leveled 12 hr after 2-ME treatment, whereas $\Delta\Psi_m$ continually decreased for 24 hr.

Effect of antioxidants on 2-ME-induced apoptosis

Because the increase of ROS and the decrease of mitochondrial transmembrane potential occurred at the same time, it is not clear whether the decrease in $\Delta\Psi_m$ is a consequence of mitochondrial damage by provoked ROS (initial ROS-formation) or whether ROS-generation is the consequence of the breakdown of the respiratory chain (secondary ROS-formation).

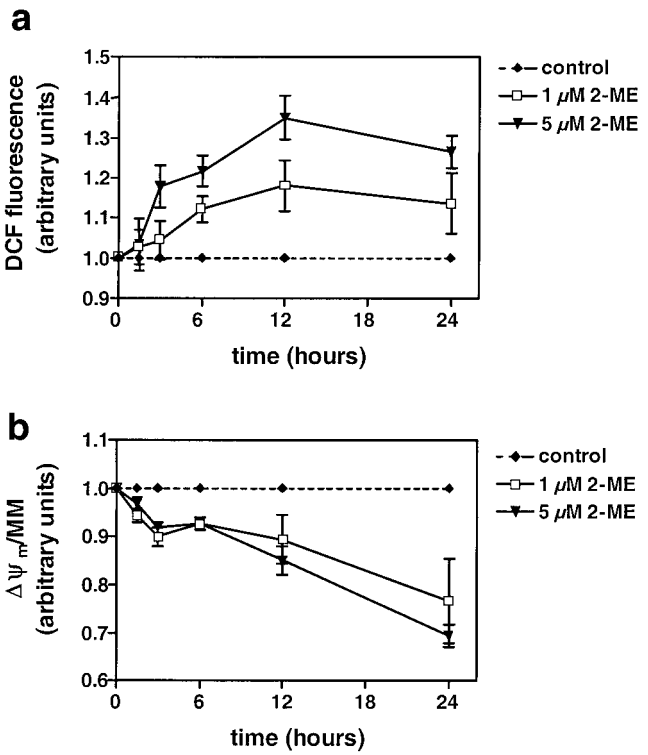

FIGURE 1 – Enhancement of ROS-formation and decrease in mitochondrial transmembrane potential ($\Delta\Psi_m$) after 2-ME treatment. Cells were treated with 0.1% ethanol (control), 1 or 5 μM 2-ME for 1.5, 3 and 6 hr. (*a*) Determining ROS: cells were stained with H_2-DCF-DA and analyzed by flow cytometry. (*b*) Cells were stained with Rhodamine 123 for measurement of $\Delta\Psi_m$ or stained with nonyl-acridine-orange to determine mitochondrial mass (MM). The quotient of $\Delta\Psi_m$/MM was calculated to correct $\Delta\Psi_m$ for differences in mitochondrial mass. Data represent means ± SEM of at least 3 independent experiments.

We investigated the effect of antioxidants on 2-ME anti-tumor efficacy. In these experiments, DS-sarcoma cells were incubated with 6 different antioxidants 4 hr before 2-ME addition. The following antioxidants were tested: α-tocopherol (vitamin E, 25 μM), ascorbic acid (vitamin C, 10 μM), butylated hydroxytoluene (BHT, synthetic fat-soluble ROS-quencher, 25 μM), N-acetyl-L-cysteine (glutathione precursor, 5 mM), mannitol (scavenger of hydroxyl radicals, 3 mM) [25] and the drug ambroxol (100 μM), which detoxifies superoxide anions.[26] In a previous study we showed the kinetics of DNA degradation after 2-ME treatment.[14] Based on these facts, we chose an incubation time of 72 hr, after which cells were harvested and analyzed for DNA fragmentation. A significant reduction ($p < 0.05$) of ROS formation was observed only by vitamin C, α-tocopherol and BHT (Fig. 2*a*). None of the used antioxidants could block DNA fragmentation completely, however, vitamin C and NAC are likely to have a moderate protective effect that is apparent in preservation of the integrity of DNA at the high molecular weight region (Fig. 2*b*, Lanes *3*,*5*). Because we wanted to compare DNA-fragmentation with colony forming efficiency (CFE), the concentration of 2-ME was reduced to 0.35 μM because higher concentrations of 2-ME prevented colony formation. The moderate protective effect of vitamin C and NAC observed on DNA fragmentation could not be confirmed by CFE-assay. No statistically significant differences in colony formation of 2-ME treated DS-sarcoma cells with or without addition of antioxidants were determined (data not shown).

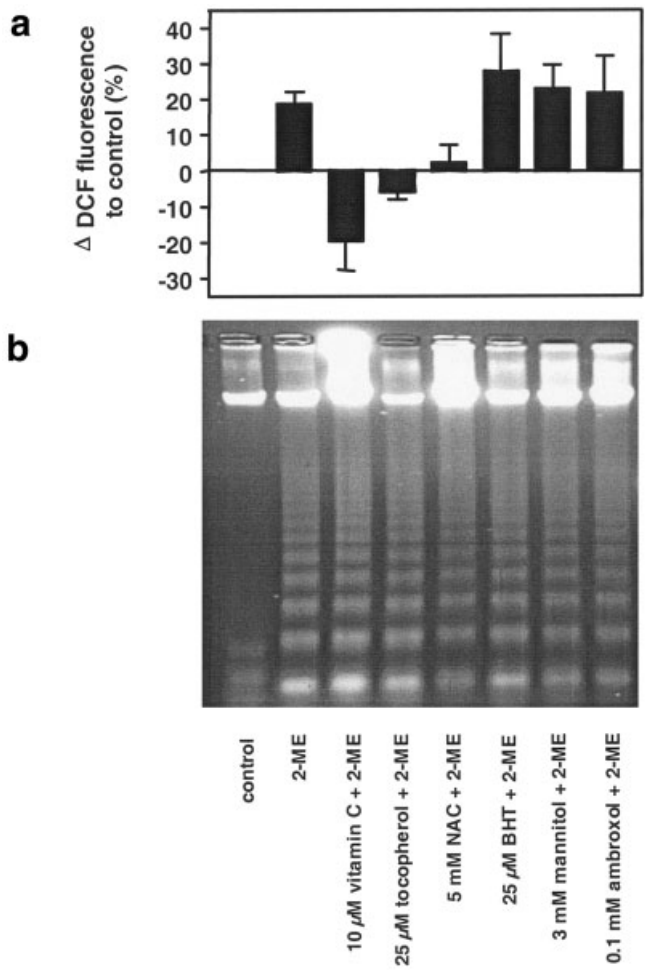

FIGURE 2 – Influence of antioxidants on 2-ME-induced ROS formation and DNA fragmentation. (*a*) Inhibition of 2-ME mediated ROS-formation through vitamin C, E and BHT. DS-sarcoma cells were treated with the indicated antioxidants for 4 hr. 2-ME at a final concentration of 5μM was then added for further 12 hr. ROS formation was determined by H_2-DCF staining and flow cytometric analysis. Baseline shows ROS formation of control cells. (*b*) Effect of antioxidants on 2-ME caused DNA fragmentation. 4 hr before 2-ME addition (0.35 μM) cells were treated with antioxidants. After 72 hr of 2-ME incubation DNA fragmentation was visualized by gel electrophoresis of genomic DNA (*b*). Data presented in (*a*) are means ± SEM of 4 independent experiments.

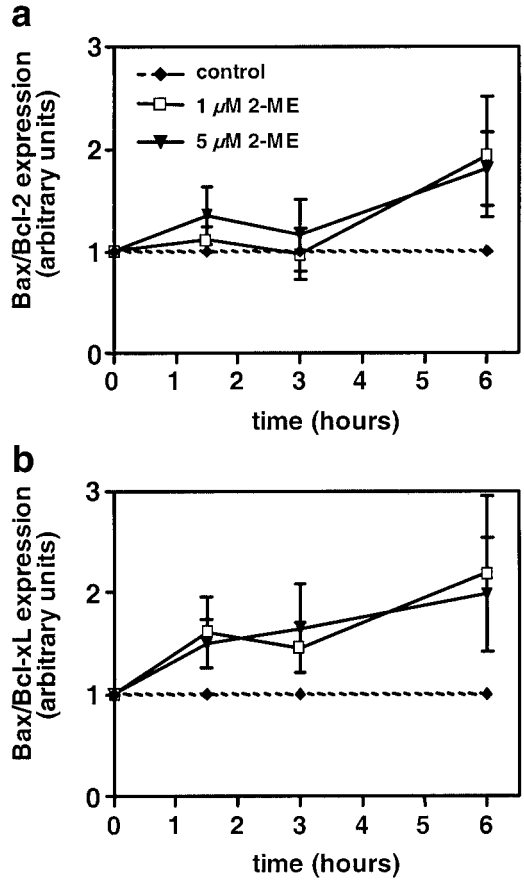

FIGURE 3 – 2-ME treatment increased portion of pro-apoptotic Bax in comparison to anti-apoptotic Bcl-2 and Bcl-x_L. DS-sarcoma cells were incubated with 0.1 % ethanol (control), 1 or 5 μM 2-ME for the indicated periods. Subsequently, cells were lysed, mitochondria were isolated and Western blotting was carried out (10 μg protein). Relation of densitometric values of Bax and Bcl-2 (*a*), and Bax and Bcl-x_L (*b*) were calculated. Ratio of control cells were set at 1. Data presented are means ± SEM of 3 independent experiments.

Bcl-2 proteins

To understand the mechanism by which 2-ME damages mitochondria, we investigated the status of anti- and pro-apoptotic proteins. We quantified the expression of pro-apoptotic Bax and Bid as well as anti-apoptotic Bcl-2 and Bcl-x_L in mitochondrial cell fractions using Western blotting. In Figure 3, the time course of Bax to Bcl-2 ratio (Fig. 3*a*) and Bax to Bcl-x_L ratio (Fig. 3*b*) in mitochondrial fractions of 2-ME treated DS-sarcoma cells demonstrates a relative increase in pro-apoptotic Bax. Bax to Bcl-x_L ratio increased within 1.5 hr after the addition of 2-ME, whereas the ratio of Bax to Bcl-2 rose to a lesser extent. Six hours after 2-ME addition (1 and 5 μM 2-ME), the Bax/Bcl-2 ratios were significantly enhanced ($p < 0.01$). The same tendency was seen for the Bax/Bcl-x_L ratios (no significance). The increase in Bax/Bcl-x_L ratios 1.5 hr and 3 hr after 2-ME treatment was due to an increase in Bax. In contrast, the relative consistent Bax/Bcl-2 ratio observed at these time points could be attributed to a simultaneous enhancement of Bax and Bcl-2. Six hours after treatment the amount of Bax was reduced to control level whereas Bcl-2 and Bcl-x_L decreased. Therefore, the Bax/Bcl-2 and Bax/Bcl-x_L ratios increased. For Bid expression, we could neither detect a regulation in whole cell lysates nor in the mitochondrial fractions of 2-ME stimulated DS-sarcoma cells (data not shown).

Release of mitochondrial nucleases

The increase in pro-apoptotic Bax molecules causes a destabilization of the outer mitochondrial membrane that results in release of proteins located in the mitochondrial intermembrane space. Apoptosis inducing factor (AIF) and the recently discovered endonuclease G (endoG) are two nucleases that are located in this space. Release of these nucleases into the cytosol results in DNA laddering, with AIF inducing large-scale fragmentation (>50 kbp) and endoG inducing nucleosome-size fragmentation.[27] As shown in Figure 4, both nucleases were released from mitochondria after 2-ME treatment. Increase in cytosolic AIF was up to 5.8-fold within 72 hr after addition of 1 μM 2-ME and 9.0-fold after addition of 5 μM 2-ME (Fig. 4*a*). Because endoG could not be assessed in the cytosol of 2-ME treated DS-sarcoma cells, it was determined in its target region, the nucleus. There, a clear accumulation of endoG protein was observed after 2-ME treatment (Fig. 4*b*). EndoG seemed to be discharged earlier than AIF, however, AIF was released to a greater extent.

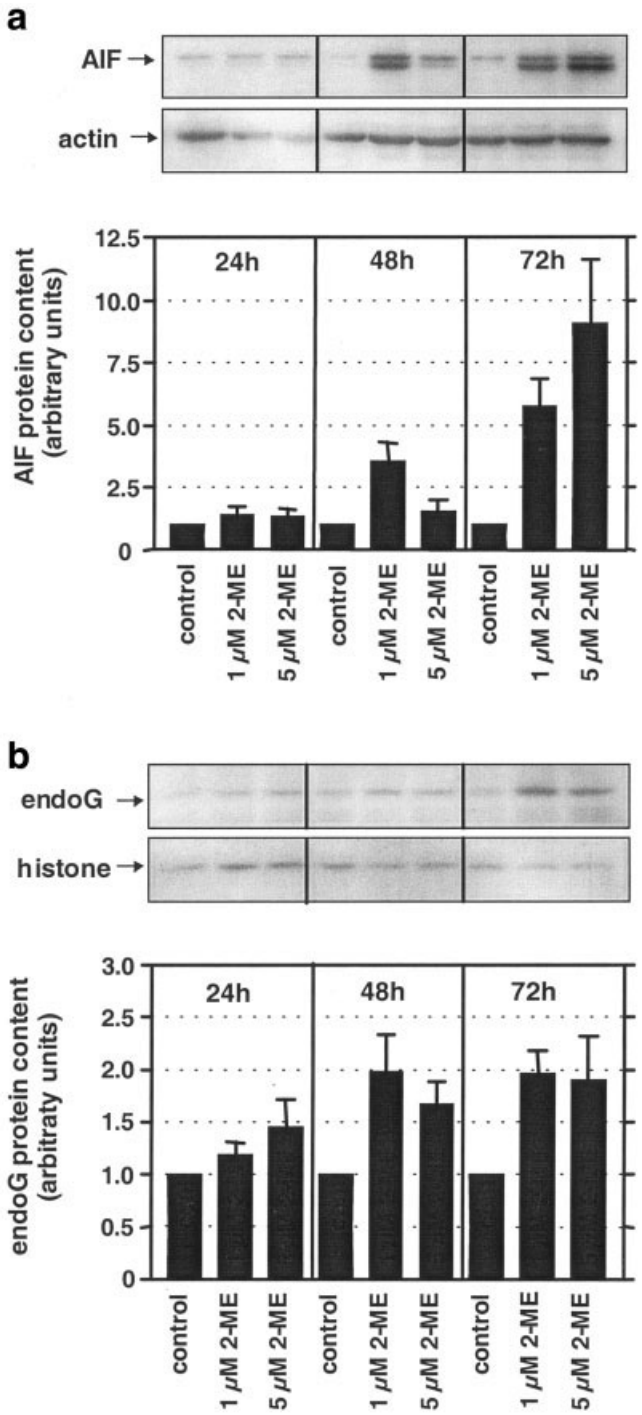

FIGURE 4 – Time course of the mitochondrial release of AIF and endoG. DS-sarcoma cells were incubated with ethanol (control), 1 or 5 μM 2-ME for 24, 48 and 72 hr. (*a*) Cytosolic fractions (10 μg protein) were subjected to immunoblot analysis with AIF antibody or (*b*) nuclear fractions (15 μg protein) with endoG antibody. Actin (cytosolic fraction) and histone H2B (nuclear fraction) were used as internal controls. Data presented are means ± SEM of 3 independent experiments.

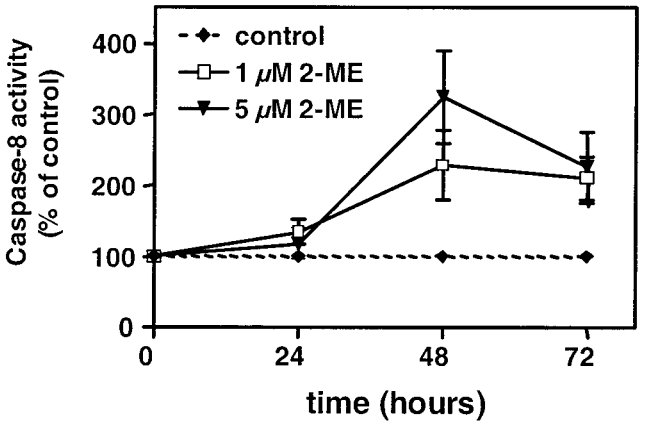

FIGURE 5 – 2-ME treatment induced caspase-8 activation. Cells were incubated with the indicated 2-ME concentrations for 24, 48 or 72 hr. Thereafter, cells were washed and lysed. Caspsase-8 activity was determined using the fluorogenic substrate Ac-IETD-AMC. Data represent means ± SEM of 3 independent experiments.

Role of caspases in 2-ME-induced apoptosis

In addition to the mitochondrial changes observed in cells undergoing apoptosis, the activation of caspases is also a common phenomenon. We reported recently that administration of 2-ME to DS-sarcoma cells resulted in a 5-fold increase of caspase-3-like activity.[14] There are several mechanisms that can account for the increase in capsase-3-like activity, including activation of upstream caspases by ligand binding to death receptors (caspase-1, -2, and -8) as well as release of cytochrome c out of mitochondria (caspase-9). To investigate the role of death receptor signaling in 2-ME-induced apoptosis, we determined caspase-8 activity, as caspase-8 is activated by more death receptor types than caspase-1 and -2. DS-sarcoma cells were treated with 2-ME and analyzed for caspase-8 activation by measuring the cleavage of a fluorogenic caspase-8 substrate. The temporal change of caspase-8 activity (Fig. 5) showed a maximum at 48 hr, where it was increased 2.3-fold (1 μM 2-ME) and 3.2-fold (5 μM 2-ME) relative to control cells and remained elevated even after 72 hr.

We next studied the cellular events leading to caspase-8 activation by monitoring the expression of 2 death receptor ligands, FasL and TNFα through RT-PCR. FasL and TNFα are ligands of CD95 (Fas) and TNF-R1, two death receptors that can activate caspase-8 after ligand binding. As shown in Fig.6A/B, mRNA levels of FasL and TNFα were upregulated in DS-sarcoma cells 24 hr after addition of 2-ME. Administration of 1 μM 2-ME increased FasL expression 3.1-fold and TNFα expression 3.0-fold, whereas higher concentrations of 5 μM 2-ME caused only a 2.3- and 1.9-fold upregulation in FasL/TNFα expression, respectively. At 48 hr after 2-ME treatment, FasL and TNFα expression declined.

To assess the relevance of these results with respect to the role of caspase-3 activation in the apoptotic process of 2-ME treated DS-sarcoma cells (FasL and TNFα confer caspase-8 activation and finally caspase-3 activation), we studied the effects of pan caspase inhibitor Z-VAD-FMK. DS-sarcoma cells were treated with 40 μM Z-VAD-FMK for one hr prior 2-ME addition, but was unable to block 2-ME-induced cell death and DNA fragmentation (Fig.7), although caspase-3-like activity and PARP-cleavage (data not shown) were blocked by this treatment.

DISCUSSION

The present study demonstrates that the ROS-formation is an early, dose-dependent event in 2-ME treated DS-sarcoma cells and this is accompanied by a decrease in mitochondrial transmembrane potential. As the ROS-formation coincided with mitochondrial breakdown, the causal relationship of these events in the induction of apoptosis cannot be unambiguously established. Three of six antioxidants examined, however (vitamin C, α-tocopherol, and

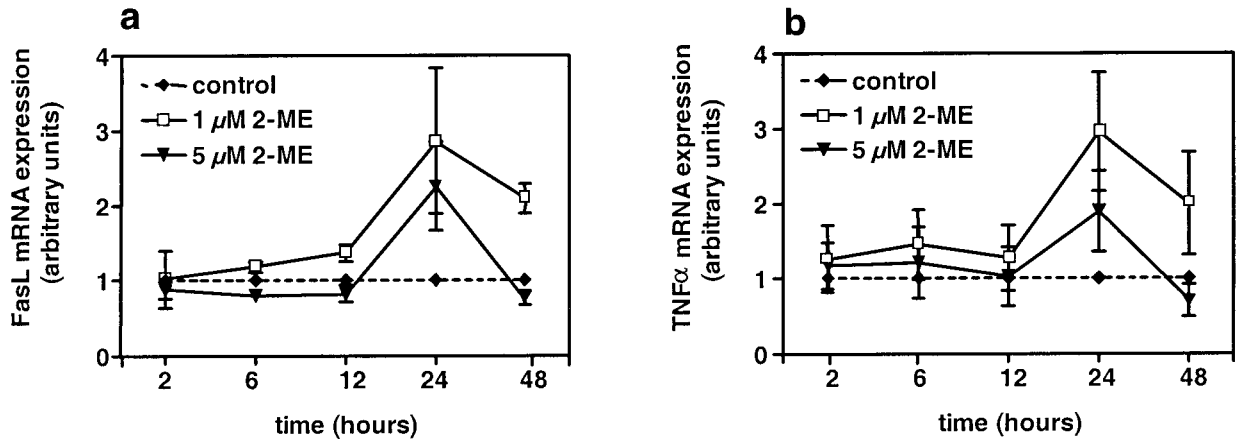

FIGURE 6 – Time course of FasL and TNFα mRNA expression after 2-ME treatment. Cells were treated with 0.1 % ethanol (control), 1 or 5 μM 2-ME for the indicated periods. Subsequently, total RNA was isolated and reversed transcribed into cDNA. FasL and TNFα mRNA expression was quantified by real time RT-PCR. GAPDH expression was used for correcting the cDNA amount. Data shown are means ± SEM of 3 independent experiments. PCR analysis of each experiment was carried out in duplicates.

NAC), quenched 2-ME-induced ROS. Among these, only vitamin C and NAC had a moderate protective effect on DNA fragmentation, which did not result in enhanced colony formation. This finding is in agreement with data of Lin *et al.*[9] who reported that 2-ME-induced apoptosis in the hepatoma cell line HepG2 could not be inhibited by the antioxidant magnolol when used at a nontoxic concentration. This contrasts with apoptosis in 2-ME-treated leukaemia cells, however, where the antioxidants ambroxol and N-acetyl-L-cysteine do inhibit apoptosis.[7]

These opposing results indicate that there may be fundamental differences between cell types or even between various cell lines of the same cell type. This precludes broad generalizations and extension of results obtained in a particular cell context to all types of cancer cells. A possible reason for this variability may reflect the diversity of the individual gene expression pattern of a given cell line, in particular as it relates to the expression of ROS detoxifying enzymes, transcription factors and membrane receptors, for example, thereby affecting the functional consequences of ROS in 2-ME-induced apoptosis. This conclusion is supported by a recent study that assessed 2-ME-induced apoptosis in seven pancreatic cancer cell lines and showed that different expression pattern of apoptosis-relevant proteins strongly influences the cell response to 2-ME treatment.[8] In the same study, Bax translocation to mitochondria was determined in MIA PaCa-2 cells, but only 48 hr after 2-ME administration. In DS-sarcoma cells we provide a more detailed time course of this event and it appears within the first 6 hr of 2-ME exposure, explaining the breakdown of mitochondrial potential at this time. We also determined absolute amount of Bax in mitochondria and find it increased already 1.5 hr after 2-ME treatment, whereas the amount of anti-apoptotic Bcl-x_L decreased (data not shown). In contrast, Bcl-2 protein increased in mitochondria of 2-ME stimulated cells within the first 3 hr. This anti-apoptotic phenomenon was also reported for 2-ME-treated endothelial cells.[13] Differences in the amount of Bcl-proteins in mitochondria after 2-ME treatment were caused by translocation of these proteins from cytosol to mitochondria, because Western blot analysis of whole cell lysates did not show any changes in Bcl-2, Bcl-x_L and Bax expression (data not shown).

The mechanism responsible for Bax integration in the outer mitochondrial membrane and the resulting changes in it is not fully understood. Before the death signal, Bax and other pro-apoptotic Bcl-2 family members localize to cytosol or cytoskeleton, whereas anti-apoptotic members like Bcl-2 and Bcl-x_L are mitochondrial integral membrane proteins. After a death signal, several events occur at the Bax protein level, including dimerization, interaction with Bid/tBid,[28,29] change in conformation that leads to the unmasking of its NH_2-terminal domain,[30] and integration in the outer mitochondrial membrane. Once integrated, Bax changes membrane integrity, for which several mechanisms have been proposed. Because of its structural similarities to the channel forming domains of the bacterial toxins colicins and diphtheria toxins, Bax (as well as Bcl-2 and Bcl-x_L) is able to form ion channels, at least in synthetic lipid membranes.[31,32] Additionally, Bax may stimulate the opening of the permeability transition pore (PTP) of the inner mitochondrial membrane through interaction with the adenine nucleotide translocator.[33] As a result of PTP opening, mitochondria would swell, leading to rupture of the outer mitochondrial membrane and passive release of cytochrome c.[34] In our experiments, mitochondrial swelling was observed in 2-ME treated DS-sarcoma cells by staining the cells with nonyl-acridine-orange. In addition to PTP, Bax is also discussed to modulate the voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane, but the underlying mechanism is not known. Shimizu *et al.*[35] reported that Bax (and Bak) bind to the VDAC of the outer membrane, leading to its opening and subsequent release of cytochrome c. Opposed effects of Bax are also discussed, however, based on the finding that the anti-apoptotic protein Bcl-x_L inhibits VDAC closure after an apoptotic signal.[36] Despite of the many uncertainties, Bax and Bcl-x_L/Bcl-2 represent mutually antagonistic players in the mechanisms converging on apoptosis.

A consequence of the destabilization or rupture of the outer mitochondrial membrane by Bax is the release of proteins localized in the transmembrane space.[37] AIF is a mitochondrial intermembrane space protein that migrates to the nucleus and participates in the induction of large-scale DNA fragmentation (fragments of approximately 50 kbp) and chromatin condensation in a caspase-independent way.[38] Our study demonstrates that 2-ME administration induces AIF release from mitochondria. In DS sarcoma cells treated with 2-ME, however, this event may not be of significant impact because we only observed low-scale, nucleosome-size DNA fragmentation (~200 bp). This is generally caused by the nuclease DFF40/CAD that is activated by caspases-3 and -7.[39] The latter can be inhibited by the non-selective caspase inhibitor Z-VAD-FMK, but this does not prevent DNA laddering in 2-ME-treated cells, suggesting that other mechanisms can mediate the observed DNA laddering. One such mechanism may be the release of endoG, which Li *et al.*[40] identified recently to cause low scale DNA fragmentation; thus endoG represents an alternative pathway to DFF40/CAD.

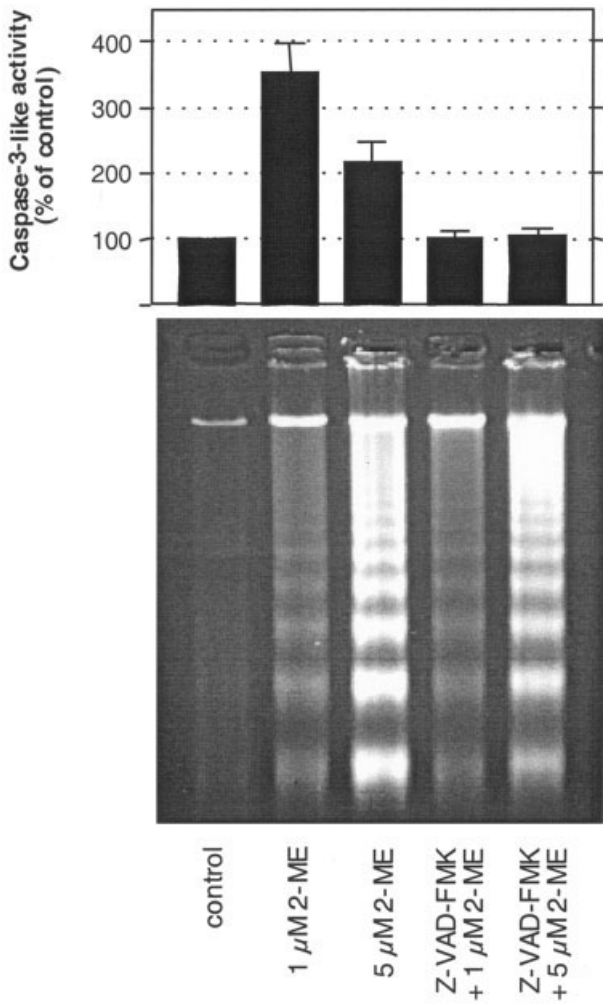

FIGURE 7 – Pan caspase inhibitor Z-VAD-FMK prevented caspase-3 activation but did not block 2-ME-induced DNA laddering. One hour before 2-ME treatment pan caspase inhibitor Z-VAD-FMK was added at a concentration of 40 μM. Control cells were supplemented with 0.1 % (v/v) ethanol and 0.4 % (v/v) DMSO (solvent of inhibitor). Caspase-3-like activity was determined 48 hr after 2-ME treatment, whereas DNA fragmentation was analyzed after 72 hr.

Mechanisms leading to the release of endoG are either formation of tBid or the action of Bim.[40] Cleavage of Bid to tBid could be induced by caspase-8,[41] granzyme B[42] or by lysosomal proteases.[43] Our investigations concerning Bid showed no significant changes in either whole cell lysates or in mitochondria. A further molecule that modulates the release of endoG is Bim, a member of the BH3 only subgroup of Bcl-2 family proteins, whose apoptotic activity seems to be independent of caspase activity and is regulated through its dissociation from microtubular dynein motor complex during apoptosis.[27] Data of Bouillet *et al*.[44] point at the relevance of Bim in the cytotoxicity of anti-microtubule agents. $Bim^{-/-}$ cells treated with the anti-microtubule drug Taxol survived 10–30 times better than Taxol-treated wild-type cells. Because 2-ME is known to increase the insoluble polymerized fraction of cellular tubulin similar to Taxol,[6] it is possible that Bim is involved in 2-ME-induced apoptosis. Reasons for the considerably stronger release of AIF compared to endoG could be (*i*) a higher concentration of AIF in the mitochondrial transmembrane space and (*ii*) different molecular mechanisms responsible for AIF and endoG release.

Although caspase-8 and caspase-3 were activated by 2-ME, experiments with the pan caspase inhibitor Z-VAD-FMK showed that 2-ME-induced cell death is caspase-independent in DS-sarcoma cells. Caspase-independent apoptosis is a well known phenomenon in the literature.[45–49] The inhibitory effects of pan caspase inhibitors on PARP cleavage and DNA fragmentation have been well documented, however, they cannot stop cell death in all cases. One reason for these findings may be that apart from caspases, Bcl-2 family members play a prominent role in apoptosis by changing the integrity of the mitochondrial membrane,[50] an observation that is confirmed in the present study. Considering caspase activation in a sequential context of ROS-formation, $\Delta\Psi_m$ decrease and Bax translocation, caspases seem to play only a secondary role that supports the ongoing apoptosis.

The same applies to FasL and TNFα upregulation after 2-ME treatment. Compared to the changes of the Bcl-2 family proteins, it appeared relatively late. Because FasL and TNFα promoter contains NF-κB binding sites,[51,52] a possible explanation for this late event could be the indirect induction of FasL/TNFα mRNA expression by 2-ME-generated ROS. Such a mechanism was reported for hepatoma cells that enhanced FasL expression by bleomycin-produced ROS[53] and for PC12 cells by H_2O_2.[54] The time course of FasL upregulation was identical to that observed in 2-ME-treated DS-sarcoma cells.

The parallelism of TNFα and FasL mRNA expression increase may be attributed to the similarity of the promotor region of these genes. As mentioned above, both promotors can be activated by NF-κB,[51,52] as well as by AP-1[55,56] and NFAT (nuclear factor of activated thymocytes),[57] whereby in addition, all three transcription factors can be activated by ROS.[58–61] In case of a general upregulation of FasL and TNFα expression by 2-ME, the finding of Hunag[7] that 2-ME increases ROS may provide a possible explanation. That study demonstrated that 2-ME-induced apoptosis in leukaemia cells is accompanied by ROS-formation, SOD-inhibition as well as SOD mRNA upregulation and mitochondrial damage. In these cells, SOD-inhibition by 2-ME seems to be the initial event of apoptosis induction, as antioxidants are able to block 2-ME mediated apoptosis. In hepatoma cells, 2-ME toxicity was not, however, inhibited by antioxidants[9] indicating cell type specific differences in response to 2-ME. Such a difference might be due to the enhanced CD95 expression of leukaemia cells compared to hepatoma cells. Upregulation of FasL by 2-ME via ROS may cause a strong death signal in leukaemia cells. Other tumor cells feature different mechanisms of cell death (*e.g.*, Bax translocation) in which FasL upregulation probably plays only a subordinated role, as these cells express no or only low levels of CD95 on their cell membrane. Upregulation of death receptor ligands, however, may be a double-edged sword. When expressed on cytotoxic T lymphocytes, natural killer cells, monocytes and dendritic cells, they induce the apoptosis of many tumor cells, whereas their expression on tumor cells induces the apoptosis of killer cells.[62] Consequently, the effect of FasL and TNFα upregulation in DS-sarcoma cells is difficult to classify although we know that these cells express CD95 and TNF-R1 (data not shown).

In addition, the role of 2-ME in death receptor signaling has been investigated by LaVallee *et al*.,[12] who reported the upregulation of death receptor 5 (DR5) in several 2-ME-treated tumor cell lines. They also showed that transfection of a dominant negative FADD (Fas associated death domain) construct results in a severe impairment of the apoptotic response after exposure of MDA-MB-231 cells to 2-ME. This finding indicates a key role of DR5 in 2-ME-induced apoptosis of these cells, although this may not be a general mechanism for all cell types. Beside the effect on tumor cells, 2-ME treatment upregulates CD95 (Fas) expression in endothelial cells as well.[13]

In summary, our study elucidates the molecular mechanism responsible for apoptosis in 2-ME treated DS-sarcoma cells. Mitochondrial changes like Bax translocation to the outer mitochondrial membrane, the decrease in mitochondrial transmembrane

potential, and the subsequent release of AIF and endoG might be of crucial importance for apoptosis. ROS-formation that appeared very early after 2-ME administration does not seem to be necessary for apoptosis-induction, but rather constitutes a by-product of mitochondrial changes or an effect of SOD inhibition by 2-ME and plays only a minor role in the ongoing apoptosis of DS-sarcoma cells. In addition, we have shown that caspase-8 and caspase-3 are activated much later than the mitochondrial changes, suggesting that they are not essential for reaching the apoptotic end point of DNA fragmentation. Further studies are needed to understand the varying action of 2-ME in different tumor cell types, as this information will be of substantial clinical interest.

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