Title
Disulfiram induces apoptosis in human melanoma cells: a redox-related process.

Permalink
https://escholarship.org/uc/item/16d2m3qm

Journal
Molecular cancer therapeutics, 1(3)

ISSN
1535-7163

Authors
Cen, Dazhi
Gonzalez, Rachel I
Buckmeier, Julie A
et al.

Publication Date
2002

Peer reviewed
Disulfiram Induces Apoptosis in Human Melanoma Cells: A Redox-related Process

Dazhi Cen, Rachel I. Gonzalez, Julie A. Buckmeier, Ravi S. Kahlon, Nilou B. Tohidian, and Frank L. Meyskens, Jr.2

Department of Medicine and Chao Family Comprehensive Cancer Center, University of California-Irvine, Orange, California 92868

Abstract
Melanoma is highly resistant to conventional chemotherapy. We have demonstrated that redox regulation in melanoma cells is aberrant, and redox-modulating agents can induce cell apoptosis. We have currently explored the effect of disulfiram (DSF), a member of the dithiocarbamate family, on apoptosis of melanoma cells in vitro. Human metastatic melanoma cells c81-46A, c81-61, and c83-2C were treated with DSF and apoptosis measured. DSF, at a dose of 25–50 ng/ml, consistently caused a 4–6-fold increase in apoptosis. The same dose of DSF did not significantly affect apoptosis in melanocytes. Coincubation of N-acetyl-cysteine reversed the DSF-induced apoptosis. Buthionine sulfoximine (BSO), an inhibitor of γ-glutamyl-cysteine synthetase, as a single agent caused a ~2-fold increase in apoptosis when incubated with melanoma cells for 4 days. BSO slightly enhanced the level of apoptosis induced by DSF (4–10% higher than DSF alone). Intracellular glutathione was remarkably depleted with BSO treatment. DSF did not cause glutathione depletion; however, the ratio of reduced and oxidized glutathione was significantly decreased (14% of control), and N-acetyl-cysteine partially restored the ratio to 30% of control. There was a transient (2-fold) elevation of intracellular superoxide level after 24 h of DSF treatment (before the overt apoptosis). The intracellular H2O2 level progressively decreased with time. DSF decreased the mitochondrial membrane polarization in a time-dependent manner, and there was a significant inverse correlation between apoptosis and mitochondrial membrane polarization. We propose that DSF-induced apoptosis is redox related but involves a different mechanism from BSO-induced apoptosis in tumor cells. Our findings have provided new data for additional understanding of drug-induced apoptosis in melanoma cells and suggests an alternative therapeutic approach to melanoma.

Introduction
Redox regulation has played an important role in cellular function, of which growth and proliferation, apoptosis, and response to oxidative stress are among the most extensively investigated (1–5). We have demonstrated previously that melanoma cells have a decreased ability to handle oxidative stress compared with normal melanocytes, resulting in a continuous build-up of ROS (6). The intracellular superoxide level was higher in melanoma cells than in melanocytes (7). The abnormal regulation of redox in melanoma cells should serve as a target for the therapeutic and/or pathogenic studies.

Glutathione homeostasis is of particular interest, because GSH protects the cell from the toxic effects of ROS formed during melanin synthesis. GSH depletion by BSO, an inhibitor of γ-glutamyl-cysteine synthetase, enhanced the cytotoxic effect of chemotherapeutic agents such as 1,3-bis(2-chloroethyl)-1-nitrosourea, cisplatin, and melphalan (8–12) inhibited DNA synthesis or growth of melanoma cell lines in vitro (9–11, 13, 14) and prolonged survival of melanoma-bearing mice after in vivo administration (11, 13, 14). BSO alone caused significant apoptosis in tumor cells, especially neuroblastoma (15–17). The intracellular glutathione content is inversely proportional to the sensitivity of different tumor cell lines to arsenic trioxide, an apoptosis-inducing agent (18). Oxidizing a critical thiol residue in the mitochondrial membrane caused permeability transition pore opening and apoptosis (19). These findings suggest that reduced thiol groups are important for maintaining cellular function and/or mitochondrial integrity.

Dithiocarbamate induces apoptosis of thymocytes by oxidizing glutathione and creating an intracellular oxidative stress (20). Our previous experiments also showed that dithiocarbamates can induce melanoma apoptosis (7). DSF, an agent used for the treatment of alcoholism, is a member of the dithiocarbamate family. In this paper, we report the effect of DSF on apoptosis in melanoma cells and a study of the redox-related mechanism, including changes in mitochondrial membrane polarization. A comparative analysis of apoptosis and cellular glutathione content between DSF and BSO treatment was also conducted.

Received 8/7/01; revised 10/10/01; accepted 10/26/01.
1 Supported in part by Grant P30CA62203, The Waltmar Foundation, and the Chao Family Comprehensive Cancer Center.
2 To whom requests for reprints should be addressed, at Chao Family Comprehensive Cancer Center, College of Medicine, University of California, Irvine, 101 The City Drive South, Building 23, Suite 403, Orange CA 92868. Phone: (714) 456-6310; Fax: (714) 456-2240; E-mail: FLMeyske@uci.edu.

The abbreviations used are: ROS, reactive oxygen species; DSF, disulfiram; NAC, N-acetyl-cysteine; BSO, buthionine sulfoximine; GSH, reduced glutathione; GSSG, glutathione disulfide; DDC, diethylthiocarbamic acid; AV, Annexin V; PI, propidium iodide; DCF 2-7-dichlorofluorescein; HE, dihydroethidium.
Disulfiram Induces Apoptosis in Melanoma Cells

Materials and Methods

Cell Culture. Metastatic melanoma cells (c81-46A, c81-61, and c83-2C) were cultured in F-10 medium (Fisher Scientific, Pittsburgh, PA) with 5% FCS, 5% newborn calf serum, penicillin (100 units/ml), and streptomycin (0.1 mg/ml). These cell strains were derived from metastatic melanoma patients, and the number of passages used in our experiments was <10 (21). Cells were split 1–2 days before drug treatment, with a density of 4–6 × 10^5/7 ml (confluence level of ~75%). Melanocytes were processed from pooled neonatal foreskins and cultured in MCDB 153 (Sigma Chemical Co., St. Louis, MO) medium containing 2% FCS, 0.3% bovine pituitary extract (Clonetics, San Diego, CA), 10 ng/ml phorbol myristate-13-acetate, 2 mM calcium chloride, 5 µg/ml insulin, and 0.1 mM 3-isobutyl-methylxanthine (Sigma Chemical Co.). Two commercial tumor cell lines, A375 (melanoma) and DU145 (prostate cancer) were cultured in DMEM with 10% FCS. Strains were derived from metastatic melanoma patients, and the number of passages used in our experiments was <10 (21). Cells were split 1–2 days before drug treatment, with a density of 4–6 × 10^5/7 ml (confluence level of ~75%). Melanocytes were processed from pooled neonatal foreskins and cultured in MCDB 153 (Sigma Chemical Co., St. Louis, MO) medium containing 2% FCS, 0.3% bovine pituitary extract (Clonetics, San Diego, CA), 10 ng/ml phorbol myristate-13-acetate, 2 mM calcium chloride, 5 µg/ml insulin, and 0.1 mM 3-isobutyl-methylxanthine (Sigma Chemical Co.). Two commercial tumor cell lines, A375 (melanoma) and DU145 (prostate cancer) were cultured in DMEM with 10% FCS.

Drug Administration. DSF, NAC, BSO, and DCC were all from Sigma Chemical Co. Drugs were dissolved in the culture medium except that DSF was dissolved in DMSO (Sigma Chemical Co.) and added to the culture directly. Control samples were added with the same amount of medium or DMSO (volume of DMSO was <0.2%).

Apoptosis Assay. Apoptosis assay was performed according to the manufacturer’s protocol (PharMingen). Briefly, cells were trypsinized, washed twice in PBS, and resuspended in binding buffer [10 mM HEPES NaOH (pH 7.4), 140 mM NaCl, 2.5 mM CaCl2] at a concentration of 1 × 10^6 cells/ml, of which 100 µl of AV conjugated to FITC (Molecular Probes, Eugene, OR) and 10 mM PI for 15 min at room temperature. Cells were then analyzed by flow cytometry using a Becton Dickinson FACScan. The proportion of apoptotic cells was estimated by the percentage of cells that stained positive for AV while remaining impermeable to PI (AV+/PI−); necrosis was defined as positive stain with both AV and PI (AV+/PI+); and viability was defined as AV−/PI− (Fig. 1). Trypan blue stain was also used in parallel, and the number of trypan blue-positive cells was similar to the sum of AV+/PI− and AV+/PI+ cells. This method was based on the literature published previously that in the early stages of apoptosis, phosphatidylserine is translocated from the inner to the outer leaflet of the plasma membrane at the cell surface. AV has a high affinity for phosphatidylserine binding on the cell surface. In the late stage of cell death, membrane integrity is lost and taken up by PI (22).

Intracellular ROS Measurement. Hydrogen peroxide level was determined using 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (Molecular Probes, Eugene, OR). This fluorescence probe diffuses through cell membranes and is hydrolyzed by intracellular esterase to DCF, which remains trapped within cells, reacts with hydrogen peroxide, and generates the fluorescent DCF. Superoxide anion was measured using HE probe (Molecular Probes). HE is the sodium borohydride-reduced form of ethidium bromide that is permeable to viable cells, and it can be directly oxidized to ethidium bromide by superoxide anion. Cells were incubated with the probes (5 µM of DCF and 10 µM of HE) for 15 min, harvested, and analyzed by flow cytometry using a Becton Dickinson FACScan with Cell Quest software with an excitation of 488 nm (both DCF and HE) and filters of 530 nm/585 nm (DCF/HE). The fluorescence intensity is proportional to the intracellular ROS levels. The above methods were adapted from those described previously (23, 24).

Analysis of Mitochondrial Membrane Polarization. JC-1 (Molecular Probes) is a cationic dye that exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (525 nm) to red (590 nm). Consequently, mitochondrial depolarization is indicated by a decrease in red fluorescence and an increase in green fluorescence. Cells were incubated with JC-1 (3 µM) for 45 min and harvested as described above. The fluorescence is quantitated by flow cytometry and is proportional to the level of mitochondrial polarization in the cell. Data are expressed as a percentage of the control.

Quantiﬁcation of Intracellular Glutathione and GSSG. Cells grown in monolayer were trypsinized and washed twice with PBS. Viability and total viable cells were counted by trypan blue exclusion, and apoptosis assay (at 72 h of incubation) was performed as described above. Cell lysates were prepared by adding 200 µl of 10 mM HCl to the pellet, followed by freeze and thaw three times in liquid nitrogen. Lysate (40 µl) was used for protein assay, and the rest (160 µl) was protein-precipitated by adding 40 µl of 5-sulfosalicylate acid (6.5%, Sigma Chemical Co.) for 10 min on ice and centrifuged at 2000 × g, 4°C, for 15 min. Supernatants were stored at −80°C until tested. The DTNB–enzyme recycling assay protocol for total glutathione and GSSG) was performed according to methods published previously (25). Protein quantitation was performed in parallel according to the Bio-Rad Dc Protein Assay kit (Bio-Rad Laboratories, Hercules, CA).

Results

DSF-induced Apoptosis of Human Metastatic Melanoma Cells. Shown in Fig. 1 is the result of a typical experiment. Cells were treated with DSF and analyzed for apoptosis as described in “Methods and Materials.” There was a good
level of consistency in the control samples (Fig. 2). The viability was between 80–90%, and in most experiments, the sum of the control apoptotic and necrotic populations was <20%.

DSF increased the frequency of apoptosis in all three of the metastatic melanoma cells at a dose level of 25–50 ng/ml (Fig. 2). The maximum frequency of cell death (the sum of apoptosis and necrosis) ranged from 400% to 600% of the control level, and the viability from 28% to 55% of the control. A higher fraction of necrosis was observed in c83-2c, a more aggressive cell strain with the highest proliferation rate. Using trypan blue exclusion, total viable cells per culture in the DSF-treated c81-46a cells were 15.0% of the control (Table 2). DSF treatment of melanocytes caused maximum cell death and viability that were 137% and 83% of control, respectively (Fig. 2). The lowest DSF dose that caused maximum apoptosis in melanoma cell strains (in this culture system) was 50 ng/ml (0.17 μM).

To additionally test the selectivity of DSF-induced apoptosis, we have used two tumor cell lines. The melanoma cell line (A 375) was also sensitive to DSF treatment, with a maximum cell death of 1300% and viability of 36.7% of the control, respectively. A prostate cancer cell line (DU 145) was resistant to DSF treatment in vitro (cell death and viability being 119% and 96% of control, respectively). This result indicates that DSF is not a universal killing agent in vitro. We do not know if DSF-induced apoptosis is melanoma-specific, because the culture conditions between melanoma cells and melanocytes (and other tumor cells) were different.

Melanoma cell strains and melanoma cell line (A375) were both sensitive to DSF, yet they were cultured in different medium. A375 and DU 145 were cultured in the same medium (DMEM), but only A375 was significantly sensitive to DSF treatment. However, cell culture using the same medium did not mean that the cysteine content was the same between two different cell lines. Even if the cysteine content in the culture medium was the same, different cell lines may require different amount of cysteine for optimal growth. It is our plan to measure the cysteine content before and after drug treatment on the same cell line.

One major metabolite of DSF, DDC, was also active in inducing apoptosis in melanoma cells (420% of control). The lowest dose that caused maximum apoptosis was 200 ng/ml (0.888 μM; Fig. 3).

**Redox Status in DSF-induced Apoptosis.** In an attempt to study the role of thiol-redox in DSF-induced apoptosis, we have performed sequential experiments to address the central question: is glutathione depletion or oxidation an essen-
Disulfiram Induces Apoptosis in Melanoma Cells

Fig. 3. DDC, a major metabolite in vivo, caused significant apoptosis in melanoma cells. Cells (3 x 10⁶) were incubated with DDC (200 ng/ml) for 3 days and analyzed by flow cytometry with AV and PI as described in Fig. 1. Results are represented from four experiments; bars, ± SD.

Fig. 4. Coincubation of NAC with DSF reversed the effect of apoptosis and restored the viability of melanoma cells. Cells (c81-46a, 3 x 10⁶) were incubated with DSF alone (50 ng/ml) or the combination of DSF (500 μg/ml) + NAC (2 mM) for 3 days and analyzed by flow cytometry with AV and PI as described in Fig. 1. Results are represented from six experiments; bars, ± SD.

tial factor for inducing apoptosis? The addition of NAC, a glutathione precursor, significantly reversed the DSF-induced cell death (from 400% to 151% of control) and increased the viability (from 51.7% to 91.6% of control; Fig. 4).

The effect of DSF on apoptosis was compared with BSO, a GSH-depleting agent. BSO alone induced a level of apoptosis ~100% of the control, which required a 4-day incubation period (Table 1). BSO slightly enhanced the DSF-induced apoptosis (The level of apoptosis in the combination of DSF and BSO was about 4–10% higher than that treated with DSF alone; Table 1). On the basis of the finding that DSF caused a higher frequency of cell death than BSO-treated melanoma cells, we additionally analyzed the intracellular total glutathione and GSSG level, and compared the level with the viability/apoptosis. As shown in Table 2, total glutathione was depleted 24 h after BSO treatment, and only slight changes of cell viability and total viable cells were observed (95.5% and 97.7% of control, respectively). Treatment of melanoma cells with BSO for 72 h caused additional depletion of cellular glutathione, and viability was relatively maintained (93.8% of control); however, total viable cell number decreased to 57.4% of control. DSF did not deplete cellular glutathione after either 24 or 72 h of treatment, and the coincubation of NAC did not increase the glutathione level despite the fact that DSF caused significant cell death at 72-h incubation (viability, total viable cells, and apoptosis + necrosis were 53.4%, 15.0%, and 365.2% of control, respectively), and NAC reversed the DSF-induced effect. We additionally measured the cellular content of GSSG at 24 h of drug treatment. There was a significant increase in the GSSG level in the DSF-treated melanoma cells (727% of control), and the ratio of GSH:GSSG was decreased to 14% of control. NAC partially restored the ratio to 30% of control (Table 2). The GSSG level in BSO-treated cells was too low to be detected. It should be noted that the glutathione levels after 24 h of DSF incubation are the more reliable measurement of GSH/GSSG contents in viable cells because of higher frequency of cell death at 72-h incubation.

DSF-induced Apoptosis Was Associated with Decrease in Mitochondrial Membrane Potential. (Fig. 5). Melanoma cells (c83-2c) were incubated with DSF (50 ng/ml) for a time course of 4–72 h, and apoptosis/necrosis and mitochondrial membrane potential were measured accordingly. There was a progressive decrease of membrane polarization with increasing apoptosis level during the course of DSF treatment with a significant inverse correlation ($r = -0.9838; P = 0.0025$). The inverse correlation between total cell death (apoptosis + necrosis) and mitochondrial membrane polarization was also significant ($r = -0.8930; P = 0.0413$).

DSF Caused a Transient Elevation of Cellular Superoxide Anion Level. Melanoma cells (c83-2c) were incubated with DSF (50 ng/ml) for a time course of 4–72 h, and intracellular ROS levels were measured. DSF caused a transient (2-fold) increase in the intracellular superoxide level at 24 h of incubation before overt apoptosis was observed. The intracellular $H_2O_2$ level decreased progressively over time to ~10% of the control values (Fig. 6).

Discussion

The abnormal redox regulation in melanoma cells may provide a candidate target for therapeutic purpose. Dithiocarbamate, as a redox-regulating agent, can induce apoptosis in melanoma cells (7). In this study, we demonstrated that DSF caused a consistent increase in the level of apoptosis (about 400–600% of control) in all three of the melanoma cell strains being tested at a very low dose (50 ng/ml; 0.17 μM). This (in vitro) dose of DSF was ~12.5% of the serum concentration, when taking DSF p.o. at therapeutic dose (500 mg/day). The same dose of DSF did not have a significant apoptotic effect on melanocytes and the prostate cancer cell line DU 145. We are currently testing more tumor cell lines with DSF and studying the selectivity of DSF-induced apoptosis. Melanoma is largely resistant to conventional chemotherapy (26–31). New agents (or combinations) need to be developed for this refractory malignancy. The advantages of using DSF for this study, apart from being a redox-regulating agent, are several-fold. DSF is an oral agent that is convenient to administer; the pharmacokinetic status and toxicity profiles are well known; and it can cross the blood brain barrier.
The percentage of viability/apoptosis did not change significantly, the total number of viable cells (57.4% of control), although Treatment of melanoma cells with BSO for 72 h decreased fold of control level) in the melanoma cell strains, and this treatments showed that BSO alone could induce apoptosis (2- effect and restored the viability (Fig. 4). These results suggested that glutathione redox balance contributed more to the DSF-induced apoptosis than glutathione depletion per se. The cellular GSSG level in BSO-treated melanoma cells was too low to be measured because of a significant depletion of total glutathione. Although the combination of DSF and BSO resulted in only slight increase in apoptosis than DSF alone, the long-term effect of this combination is not known.

Despite a higher level of apoptosis observed in DSF-treated melanoma cells, the total cellular glutathione level was not decreased. It is worth noting that NAC significantly reversed the DSF-induced apoptosis and increased the melanoma cell viability without increasing the cellular GSH level. Additional measurement of cellular GSSG, the oxidized form of glutathione, showed that DSF treatment significantly increased the GSSG content (727% of control). The ratio of GSH:GSSG was significantly decreased (14% of control), and NAC partially restored the ratio to 30% of control. These results suggested that glutathione redox balance contributed more to the DSF-induced apoptosis than glutathione depletion per se. The cellular GSSG level in BSO-treated melanoma cells was too low to be measured because of a significant depletion of total glutathione. Although the combination of DSF and BSO resulted in only slight increase in apoptosis than DSF alone, the long-term effect of this combination is not known. In vivo study (using this drug combination) to monitor the kinetics of tumor progression may provide additional information.

Glutathione depletion may not be the sole factor in the observed apoptosis. Sato et al. (35) had reported using Jurkat T cells and human peripheral blood lymphoblasts as the target for BSO and diamide treatment. Depletion of cellular barrier with potential efficacy for central nervous system metastasis.

The effects of DSF on cellular redox changes have not been well studied. Dithiocarbamate induces apoptosis in rat thymocytes and astrocytes (20, 32) by chelating copper and creating an intracellular oxidative stress by oxidizing and/or depleting cellular glutathione. DSF binds to proteins via formation of mixed disulfides with free thiol groups of various proteins (33), and it may affect mitochondrial membrane proteins, which are redox sensitive (34). Disturbance in the glutathione redox may affect cellular ROS scavenging. We have performed sequential experiments to address the above questions. We coincubated NAC with DSF-treated melanoma cells. NAC significantly reversed the apoptotic effect and restored the viability (Fig. 4). These results suggested that the pool of glutathione or another thiol redox buffer is involved in the process of apoptosis induced by DSF. However, we do not know if cysteine in the medium interacts with DSF.

The redox-related apoptosis in melanoma cells induced by DSF was also compared with BSO treatment. Our experiments showed that BSO alone could induce apoptosis (2-fold of control level) in the melanoma cell strains, and this level of apoptosis required a 4-day treatment with BSO. Treatment of melanoma cells with BSO for 72 h decreased the total number of viable cells (57.4% of control), although the percentage of viability/apoptosis did not change significantly (Tables 1 and 2). The BSO-induced apoptosis was associated with a significant (>90%) glutathione depletion (Table 2). The above result was consistent with the findings of other investigators that BSO is an active agent for inhibiting tumor cell growth and inducing apoptosis (9, 10).

Table 1  The effect of BSO and DSF on apoptosis

<table>
<thead>
<tr>
<th></th>
<th>Day 3 (n = 3)</th>
<th>Day 4 (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Viability</td>
<td>Apoptosis</td>
</tr>
<tr>
<td></td>
<td>Cont BSO DSF DSF + BSO Cont BSO DSF DSF + BSO</td>
<td></td>
</tr>
<tr>
<td>% Total</td>
<td>87.157 ± 0.254 85.580 ± 1.140 53.273 ± 1.156 43.747 ± 1.810 86.313 ± 1.318 72.128 ± 2.773 33.525 ± 5.219 21.997 ± 3.026</td>
<td></td>
</tr>
<tr>
<td>p</td>
<td>0.268 0.0014 0.0021</td>
<td>0.034 0.0037 &lt;0.0001</td>
</tr>
<tr>
<td>Necrosis</td>
<td>5.807 ± 0.44 6.097 ± 0.76 24.703 ± 0.28 28.853 ± 0.378 5.887 ± 0.69 11.823 ± 1.24 24.435 ± 2.17 26.047 ± 3.15</td>
<td></td>
</tr>
<tr>
<td>p</td>
<td>0.683 0.0012 0.0001</td>
<td>0.047 0.0074 0.0008</td>
</tr>
</tbody>
</table>

* Statistically significant when compared with control, analyzed by t test.

Table 2  Intracellular glutathione content of melanoma cells

<table>
<thead>
<tr>
<th></th>
<th>24 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Total Glutathione (nmol/mg protein)</td>
<td>59.4 ± 2.4</td>
<td>77.0 ± 14.0</td>
</tr>
<tr>
<td>GSH:GSSG&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.3 ± 0.5</td>
<td>9.7 ± 2.0</td>
</tr>
<tr>
<td>% Viability (Trypan blue)</td>
<td>91.700 ± 1.445</td>
<td>91.5 ± 1.99</td>
</tr>
<tr>
<td>Total viable cells (10&lt;sup&gt;6&lt;/sup&gt;)</td>
<td>2.202 ± 0.186</td>
<td>1.677 ± 0.162</td>
</tr>
<tr>
<td>% Dead cells (AV&lt;sub&gt;I&lt;/sub&gt;/PI&lt;sub&gt;G&lt;/sub&gt; plus AV&lt;sub&gt;II&lt;/sub&gt;/PI&lt;sub&gt;I&lt;/sub&gt;)</td>
<td>16.104 ± 1.664</td>
<td>58.8 ± 2.08</td>
</tr>
</tbody>
</table>

<sup>a</sup> Average of two experiments because of significant cell death and not enough protein for accurate quantitation of GSH in one sample. The exact values of cellular GSH of those two experiments were 68.39 and 78.96, respectively.

<sup>b</sup> GSH content is calculated as total glutathione – (2 × GSSG).
GSH with BSO failed to induce apoptosis despite a marked decrease of cellular GSH, which was greater than that observed in apoptosis induced by diamide. Thioredoxin was oxidized by incubation of these cells with diamide, suggesting that other cellular thiols may be involved in the diamide-induced T-cell apoptosis. Spyrou and Holmgren (36) also reported that BSO (0.1 mM) rapidly stopped GSH synthesis in exponentially growing 3T6 mouse fibroblasts, but the GSH depletion did not significantly affect the cell growth, DNA synthesis, and the pools of deoxyribonucleoside triphosphates, suggesting that GSH-glutaredoxin system is not the sole hydrogen donor system for ribonucleotide reductase. It is well known that thioredoxin and its reductase provides reducing equivalents for the cellular proteins and metabolites, including ribonucleotide reductase (37-40). The disturbance of glutathione balance in DSF-treated melanoma cells may reflect only one aspect of the thiol-redox alterations. Other components in the thiol-redox buffer system (such as thioredoxin and thioredoxin reductase, glutaredoxin and glutathione reductase) are also operating to maintain the cellular redox homeostasis. There are at least two major intracellular disulfide-reducing systems, the glutathione system and the thioredoxin system (38), and the latter one is regarded as an essential constituent of the cell-proliferating machinery in mammalian cells. It is our plan to additionally explore the role of those components on the DSF-treated melanoma cells.

The lack of significant DSF/BSO effect (as compared with DSF alone) can be attributable to maximal depletion of GSH by BSO. We have performed sequential incubation experiments (BSO followed by DSF) resulting in a more decreased apoptosis levels than coincubation treatments (data not shown). We have also used different doses of BSO (coincubation with DSF), and there were no significant changes in the apoptosis level.

We cannot exclude the possibility that DSF and NAC may affect the results of glutathione reductase-recycling assay. It has been reported that DSF may interfere with such an assay (41). Our next experiment is to incubate melanoma cells with DSF (or NAC) for 24 h and determine the intracellular uptake (content) of DSF (or NAC) and assay of GSH/GSSG by mixing untreated (control) cell lysate with known intracellular dose of DSF (or NAC) and compare the results with the untreated cells.

In this study, we also tested whether the redox-related apoptosis induced by DSF is associated with a decrease in the mitochondrial membrane potential in melanoma cells. Our finding showed that there was a significant inverse correlation of mitochondrial membrane polarization and the apoptosis/total cell death during the course of DSF incubation.

---

**Fig. 5.** DSF-induced apoptosis is associated with decrease in mitochondrial membrane potential. Cells (c83-2c) were incubated with DSF (50 ng/ml) for 4, 24, 48, and 72 h and analyzed for mitochondrial membrane potential (with JC-1 probe, as described in "Materials and Methods"), apoptosis, and total cell death (apoptosis + necrosis, as described in Fig. 1). Correlation between mitochondrial membrane potential and cell death (at corresponding time point) is illustrated in Fig. 5B. Results are expressed as the percentage of the control.

---

**Fig. 6.** DSF caused a transient increase in the cellular superoxide level. Cells (c83-2c) were incubated with DSF (50 ng/ml) for 4, 24, and 72 h, and analyzed for intracellular H₂O₂ (A) and superoxide (B) by 6-carboxy-2′-7′-dichlorodihydrofluorescein diacetate and HE, as described in "Materials and Methods." Shown in parentheses (9) are viabilities (preformed by trypan blue exclusion). Results are represented from three experiments; bars, ± SD.
We hypothesize that changes in mitochondrial membrane potential might reflect apoptotic effects more accurately than late events of cell death (loss of membrane integrity). Experiments with other cell strains also showed a consistent decreased level of mitochondrial membrane polarization (approximately 40–60% of control) occurring at different time points (data not shown). The role of mitochondrion in the cell death control is being extensively investigated (42, 43). There are many different apoptosis-inducing conditions that can cause dissipation of mitochondrial ΔΨm (33). Thiold cross-linking agents such as diamide, dithiodipyridine, or bis-maleimido-hexane can cause a covalent modification of a protein located in the inner mitochondrial membrane, adenine nucleotide translocator, leading to mitochondrial membrane permeabilization and cell death, and the process is Bcl-2 independent (19). It has been reported that DSF binds to proteins both in vivo and in vitro via formation of mixed disulfides with the free thiol groups of various proteins (33), and it is likely that this binding process may affect the function of the thiold-redox buffer (as mentioned above) and/or mitochondrial membrane integrity. Whether the DSF-induced apoptosis in melanoma cells is a direct action on mitochondria or an indirect effect through cellular redox changes remains to be answered. Study on purified mitochondria can additionally dissect the mechanisms of DSF on mitochondrial redox status and apoptosis.

There was a transient increase in the intracellular superoxide level after 24 h of incubation with DSF. Multiple experiments have shown that 24-h incubation is the earliest time point for detectable cell death (using AV and trypan blue stain, the viable fraction is approximately 75–95% of the control), and the findings were supported by the kinetic studies of apoptosis and the mitochondrial membrane potential (Fig. 5). Prolonged incubation of 48–96 h caused increasing frequency of apoptosis/necrosis, and the intracellular ROS content is the mixture from both viable and dead cells, and the leaking of ROS from dead cells may contribute to the decreased level observed. Dithiocarbamates, especially DDC, are known to inhibit copper/Zn superoxide dismutase (44), and oxidation of glutathione and/or other protein thiol groups may interfere with superoxide scavenging. Superoxide dismutase has been identified as a target for the selective killing of cancer cells, and the cellular level of superoxide is associated with oxidative-induced apoptosis of some tumor cells (45–47). Whether the transient increase in the superoxide anion in melanoma cells leads directly to apoptosis is not known. A cell-permeable superoxide scavenger, Trion, can be used to study the relationship of intracellular superoxide level and apoptosis (48).

DSF undergoes extensive metabolism after gastrointestinal absorption, and one major metabolite is DDC (33, 49). Our experiments showed that DDC also demonstrated a comparable capacity of inducing apoptosis in melanoma cells, although at a higher dose level (200 ng/ml or 0.886 μM). This finding may provide useful information for the possible in vivo use of DSF.

Our study showed that DSF induced apoptosis in metastatic melanoma cells, and NAC reversed the apoptotic effect. The apoptosis is associated with a decrease in the GSH:GSSG ratio, decrease in mitochondrial membrane potential, and a transient increase in cellular superoxide level. In the future, we will additionally study the redox changes in the subpopulations (such as apoptotic, necrotic, and viable cells) when commercial fluorescence probe combinations are available. The differential mechanisms between DSF- and BSO-induced apoptosis may provide new information for selecting agents with complementary antimelanoma activity, and it will be interesting to study more drug combinations such as DSF with ciplatin, 1,3-bis(2-chloroethyl)-1-nitrosourea.

Acknowledgments

We thank Drs. John Fruehauf, Susan McNulty, and Pat Farmer for their helpful suggestions and discussions of this study and the manuscript.

References

Disulfiram Induces Apoptosis in Melanoma Cells


Disulfiram Induces Apoptosis in Human Melanoma Cells: A Redox-related Process

Dazhi Cen, Rachel I. Gonzalez, Julie A. Buckmeier, et al.

Mol Cancer Ther 2002;1:197-204.

Updated version
Access the most recent version of this article at:
http://mct.aacrjournals.org/content/1/3/197

Cited Articles
This article cites by 42 articles, 10 of which you can access for free at:
http://mct.aacrjournals.org/content/1/3/197.full.html#ref-list-1

Citing articles
This article has been cited by 10 HighWire-hosted articles. Access the articles at:
http://mct.aacrjournals.org/content/1/3/197.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.