Bcl-2 and Mn-SOD Antisense Oligodeoxynucleotides and a Glutamine-enriched Diet Facilitate Elimination of Highly Resistant B16 Melanoma Cells by Tumor Necrosis Factor-α and Chemotherapy

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Mitochondrial glutathione (mtGSH) depletion increases sensitivity of Bcl-2-overexpressing B16 melanoma (B16M)-F10 cells (high metastatic potential) to tumor necrosis factor-α (TNF-α)-induced oxidative stress and death in vitro. In vivo, mtGSH depletion in B16M-F10 cells was achieved by feeding mice (where the B16M-F10 grew as a solid tumor in the footpad) with an l-glutamine (L-Gln)-enriched diet, which promoted in the tumor cells an increase in glutaminase activity, accumulation of cytosolic l-glutamate, and competitive inhibition of GSH transport into mitochondria. L-Gln-adapted B16M-F10 cells, isolated using anti-Met-72 monoclonal antibodies and flow cytometry-coupled cell sorting, were injected into the portal vein to produce hepatic metastases. In L-Gln-adapted invasive (iB16M-Gln+) cells, isolated from the liver by the same methodology and treated with TNF-α and an antisense Bcl-2 oligodeoxynucleotide, viability decreased to ~12%. B16M-Gln+ cell death associated with increased generation of O2− and H2O2, opening of the mitochondrial permeability transition pore complex, and release of proapoptotic molecular signals. Activation of cell death mechanisms was prevented by GSH ester-induced mtGSH replenishment. The oxidative stress-resistant survivors showed an adaptive response that includes overexpression of manganese-containing superoxide dismutase (Mn-SOD) and catalase activities. By treating iB16M-Gln+ cells with a double anti-antisense therapy (Bcl-2 and SOD2 antisense oligodeoxynucleotides) and TNF-α, metastatic cell survival decreased to ~1%. Chemotherapy (taxol plus daunorubicin) easily removed this minimum percentage of survivors. This contribution identifies critical molecules that can be sequentially targeted to facilitate elimination of highly resistant metastatic cells.

The main cause of death from cancer is due to metastases that show resistance to conventional therapies (1). In practice intrinsic (innate) or acquired (adaptive) resistance to therapy critically limits the outcome of cancer patients (2). Multidrug and/or radiation resistance, which are characteristic features of malignant tumors, frequently associate with high GSH content in the cancer cells (e.g. Ref. 3). Malignant melanoma cell subsets show different degrees of multidrug and radiation resistance (4). Analysis of a Bcl-2 family of genes revealed that B16 melanoma (B16M)-F10 cells (high metastatic potential), as compared with F1 cells (low metastatic potential), overexpressed preferentially Bcl-2 (~5.7-fold) (5). B16M-F10 cells, as compared with F1 cells, show higher GSH content, higher rates of GSH synthesis, and a decrease in GSH efflux (5). GSH release from B16M-F10 cells is channeled through the multidrug resistance protein 1 and the cystic fibrosis transmembrane conductance regulator, a member of the ABC family of membrane transport proteins with structural similarities with the multidrug resistance protein 1 (6). The antiapoptotic protein Bcl-2 inhibits the release of GSH through cystic fibrosis transmembrane conductance regulator facilitating accumulation of GSH within the metastatic cells and, thereby, contributing to their resistance against cytotoxic agents (6).

Invasive B16M-F10 (iB16M) cells that survive after in vitro interaction with the vascular endothelium show a transient impairment of the mitochondrial system for GSH uptake and, in addition, a decreased activity of respiratory complexes II, III, and IV, less O2 consumption and ATP levels, higher O2/[bardi2] and H2O2 production, and lower mitochondrial membrane potential (7). This is important, because mitochondria do not synthesize GSH (8) and mitochondrial GSH (mtGSH) depletion facilitates mitochondrial membrane permeabilization, permeability transition pore complex (PTPC) opening, and the release of apoptosis-inducing molecular signals (5, 9, 10). In fact, by using in vitro diethylmaleate or monochlorobimane (as thiol-depleting agents), we observed that B16M-F10 cells with low mtGSH levels were highly susceptible to TNF-α-induced oxidative stress and death (Ortega et al. (5)). Interestingly, this effect was potentiated by Bcl-2 antisense oligodeoxynucleotides (Bcl-2-AS) (5). Nevertheless, tumor mtGSH depletion in vivo, under non-toxic conditions, will require a different approach.

l-Glutamine (l-Gln) is the most abundant amino acid in the bloodstream, accounting for 50% of the whole body pool of free amino acids (11). Many tumors exhibit a remarkable preference for l-Gln as respiratory fuel (12–14), and different studies have demonstrated that this preference correlates with increased mitochondrial phosphate-depend-
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ent glutaminase (Glnase) activity (e.g. 15–17). Tumor growth can behave as an L-Gln trap forcing a systemic loss of this amino acid (12). Providing supplemental L-Gln during cancer treatment, by reducing the incidence of gastrointestinal, neurological and possibly cardiac complications, has the potential to minimize chemotherapy- and/or radiation-related toxicity (18–21). L-Gln, preferentially formed and stored in skeletal muscle, is the principal metabolic fuel for small intestine enterocytes, lymphocytes, macrophages, and fibroblasts (22). L-Gln-enriched diets (GED) may support muscle L-Gln metabolism without stimulating tumor growth (e.g. Refs. 18 and 23). Moreover, a high rate of L-Gln oxidation may render the mitochondria more susceptible to reactive oxygen species (ROS)-mediated cytotoxicity by TNFα (10, 24). In previous studies in Ehrlich ascites tumor cells we found that L-glutamate (L-Glu) derived from L-Gln competitively inhibited GSH transport into mitochondria (23), thereby depleting selectively tumor mtGSH under in vivo conditions (23) and rendering Ehrlich ascites tumor cells more susceptible to oxidative stress-induced mediators (10). Therefore, theoretically, therapy against L-Gln-consuming malignant tumors could be improved by combining non-toxic TNF-α doses with a GED to facilitate inhibition of GSH transport into tumor mitochondria by L-Glu. However, there is no previous biochemical background on potential adaptive/beneficial effects of L-Gln supplementation in experimental animals bearing metastatic cancers. The B16M is a model widely used to study metastatic spread and tissue invasion (25), and our primary aim for the present work was to investigate molecular adaptations of B16M-F10 cells to a GED. Our results also reveal that L-Gln supplementation increases B16M-F10 cell sensitivity to TNFα-induced cytotoxicity and that this effect can be further increased by treatment with Bcl-2-AS. Nevertheless metastatic cell survivors follow an adaptive molecular response that improves their resistance to oxidative stress.

EXPERIMENTAL PROCEDURES

Culture of B16M-F10 and iB16M Cells—Murine B16M-F10 (from the ATCC, Rockville, MD) or iB16M (see below) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen), pHi 7.4, supplemented with 10% fetal calf serum (Invitrogen), 10 mM HEPES, 40 mM NaHCO3, 100 units/ml penicillin, and 100 μg/ml streptomycin (26). Cell integrity was assessed by trypan blue exclusion and leakage of lactate dehydrogenase activity (26).

Animals and Diets—Syngeneic male C57BL/6 mice (9 weeks old) from IFFA Credo (L’Arbresle, France) were fed ad libitum on a stock laboratory diet (Leticia, Barcelona, Spain) or an equivalent diet but L-Gln-enriched (15% of total dietary nitrogen from L-Gln) (23). Both diets were isonitrogenous and isocaloric. Mice were kept on a 12-h light/12-h dark cycle with the room temperature maintained at 22°C. Procedures involving animals were in compliance with the national and international laws and policies (European Economic Community Directive 86/609, OJ L 358, 1, December 12, 1987, and NIH Guide for the Care and Use of Laboratory Animals, NIH Publ. 85-23, 1985).

Local Tumor Growth—B16M-F10 cells were harvested from culture flasks by exposure to 0.02% EDTA (5 min at 37°C), washed twice in DMEM, resuspended in the same culture medium, and injected into the footpad of the right hind-limb (106 cells/20 μl). Local tumor growth was determined by measuring footpad diameter with calipers every 2 days, starting on the first day of treatment. Tumor size was calculated according to the formula: tumor diameter = (diameter of footpad with growing tumor) – (diameter of DMEM-treated contralateral footpad). Mice were observed daily for survival.

Experimental Metastases—Hepatic metastases were produced by intravenous injection of tumor cells into anesthetized mice (Nembutal, 50 mg/kg intraperitoneal) of 105 viable B16M cells suspended in 0.2 ml DMEM. Mice were sacrificed at 5 days after B16M-F10 inoculation. The livers were fixed with 4% formaldehyde in PBS (pH 7.4) for 24 h at 4°C and then paraffin-embedded. Metastasis density (mean number of foci/100 mm3 of liver detected in fifteen 10 × 10 mm2 sections per liver) and metastasis volume (mean percentage of liver volume occupied by metastasis) were determined as described earlier (27).

Isolation of in Vivo Growing B16M-F10 and iB16M Cells—Antimet-72 monoclonal antibodies and flow cytometry-coupled cell sorting were used to isolate viable B16M-F10 cells (from the tumor growing in the footpad) and iB16M cell (from metastatic foci growing in the liver) (see Ref. 7). Anti-Met-72 monoclonal antibodies, which react with a 72-kDa cell-surface protein (Met-72) expressed at high density on B16M clones of high metastatic activity, were produced as previously described (28) through syngeneic immunizations of C57BL/6J mice with clones of B16M-F10. Tissues containing tumor cells were obtained by surgical means. Cell dispersion was carried out in minced tissues by the following sequential procedure: 1) trypsinization (~25 mg of fresh tissue per milliliter in Mg2+- and Ca2+-free PBS supplemented with 0.2% trypsin plus 0.5 mM EDTA plus 5 mM glucose, 3 min at 37°C); 2) three washes in PBS; 3) collagenase digestion (in PBS supplemented with 0.5 mg of collagenase/ml plus 5 mM glucose, 5 min at 37°C) (steps 1 and 3 were performed in Erlenmeyer flasks where the gas atmosphere was O2/CO2, 19:1). Then cells were washed three times in PBS and resuspended in DMEM, and an aliquot containing 2 × 106 of B16M-F10 or iB16M cells was incubated with a predetermined excess of anti-Met-72 monoclonal antibody for 1 h on ice. After three washings with PBS, cells were incubated with fluorescein isothiocyanate-conjugated sheep anti-mouse IgF(ab)2 (Cappel Laboratories, Westchester, PA) for 1 h on ice. After another three washing steps with PBS at 4°C, cell pellets were resuspended in 1 ml of ice-cold PBS, filtered through a 44-μm pore mesh and analyzed using an EPICS ELITE (Coulter Electronics, Hialeah, FL). Fluorescent B16M cells were separately gated for cell sorting and collected into individual tissue culture chambered slides (Nalge Nunc International Corp., Naperville, IL). Then the sorted tumor cells were harvested and plated in 25-cm2 polystyrene flasks (Falcon Labware) as above.

Compartmentation of B16M-F10 and iB16M Cells—Cultured cells were harvested (see above), washed twice in DMEM, and resuspended in ice-cold Krebs-Henseleit bicarbonate medium (pH 7.4). Rapid separation of cytosolic and mitochondrial compartments, and calculation of mitochondrial and cell volumes, were performed as recently described (7).

Metabolite and Enzyme Assays—For amino acid analysis, plasma (separated from whole blood as previously described) (26) or intracellular fractions (see above) were treated for protein precipitation with 5% (w/v) ice-cold sulfosalicylic acid in 0.3 M lithium citrate buffer (pH 2.8), as previously described (29). After centrifugation, 50 μl of the supernatant were collected and injected into an LKB 4151α plus amino acid analyzer (LKB Biochrom, Cambridge, UK).

For enzyme assays isolated tumor cells were homogenized in 0.1 M phosphate buffer (pH 7.2) at 4°C (27). Gln synthetase activity was assayed by the method of Matsuno and Satoh (30). Phosphate-dependent Glnase activity was assayed as described by Matsuda et al. (31) except that L-Gln was replaced by [L-14C]Gln (250 mCi/mmol, 0.5 μCi/tube, Perkin-Elmer Life Sciences), and the total volume of the reaction mixture was 100 μl. Glutamate dehydrogenase, alanine transaminase, aspartate transaminase, and glucose-6-phosphate dehydrogenase activities were determined by standard procedures (32). γ-Glutamylcysteine synthetase and GSH synthetase activities were measured as described
Mitochondrial GSH Uptake—Isolated mitochondria were resuspended in incubation medium (100 mM sucrose, 50 mM KCl, 10 mM KH2PO4, 50 mM MOPS, 0.2 mM MgCl2, 1 mM EGTA, and 5 mM sodium succinate, pH 7.4) and used immediately after B16M cell fractionation (see above) (7) to avoid any loss of their initial GSH content. Using a variation of the technique described by Martensson et al. (8), as recently described in detail (Ortega et al. (7)), net rates of GSH uptake into the matrix were measured at 25 °C in mixtures (volume 500 µl) containing incubation medium (as above) and 6 mM (1 µCi per assay) [35S]GSH. GSH uptake was initiated by adding 20 µl of the mitochondrial suspension (final concentration 1 mg of protein per ml). Protein was determined by the Bradford method (39).

Northern Blot Analysis—Total RNA was isolated using the TRizol kit from Invitrogen (San Diego, CA) and following manufacturer’s instructions. cDNA was obtained using a random hexamer primer and a MultiScribe Reverse Transcriptase kit as described by the manufacturer (TaqMan RT Reagents, Applied Biosystems, Foster City, CA). A PCR master mix and AmpliTaq Gold DNA polymerase (Applied Biosystems) were then added containing the specific primers: forward 5′-CTTGGACATCAAGGAGGAAG; and reverse 5′-CTCCCCAGTGTACCTCCAA. Real-time quantification of the mRNA relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed with a SYBR Green I assay, and an iCycler detection system (Bio-Rad). Target cDNA was amplified as follows: 10 min at 95 °C, then 40 cycles of amplification (denaturation at 95 °C for 30 s and annealing and extension at 60 °C for 1 min per cycle). The increase in fluorescence was measured in real-time during the extension step. The threshold cycle (Ct) was determined, and then the relative gene expression was expressed as follows: -fold change = 2^-Δ(ΔCt), where ΔCt = Ct target - Ct GAPDH, and Δ(ΔCt) = ΔCt-treated - ΔCt control.

Quantitative Determination of the Mitochondrial Membrane Potential—Measurements of the mitochondrial membrane potential were performed, as recently described (5), by the uptake of the radiolabeled lipophilic cation methyl-triphenylphosphonium ([3H]TPMP, Amersham Biosciences), which enables small changes in potential to be detected (42). Energization-dependent TPMP uptake was expressed as an accumulation ratio in units of ([TPMP/mg of protein]/(TPMP/µl supernatant).

Measurement of H2O2—The assay of H2O2 production was based, as previously reported (43) on the H2O2-dependent oxidation of the homovanillic acid (3-methoxy-4-hydroxyphenylacetic acid) to a highly fluorescent dimer (2,2′-dihydroxydiphenyl-5,5′-diacetic acid), which is mediated by horseradish peroxidase (44).

Flow Cytometry—Cellular suspensions were diluted to ~250,000 cells per ml. Analysis were performed with an EPICS PROFILE II (Coulter Electronics) as previously described (45). Samples were acquired for 10,000 individual cells. Fluorochromes were from Molecular Probes (Pooqtegbouw, Leiden, The Netherlands), excepting dihydroethidium, which was from Sigma. Cell viability was determined by the fluorescent dye propidium iodide (final concentration 10 µM). O2⁻ generation was determined, in viable cells, using dihydroethidium (2 µg/ml) (46).

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nuclei) and propidium iodide (10 μM; which stains nuclei of cells with a disrupted plasma membrane) for 3 min and analyzed using a Diaphot 300 fluorescence microscope (Nikon, Tokyo, Japan) with excitation at 360 nm. Nuclei of viable, necrotic, and apoptotic cells were observed as blue round nuclei, pink round nuclei, and fragmented blue or pink nuclei, respectively. About 1000 cells were counted each time. DNA strand breaks in apoptotic cells were assayed by using a direct terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay (Roche Applied Science) and fluorescence microscopy following the manufacturer’s methodology.

Statistical Analysis—Data were analyzed by Student’s t test.

**RESULTS**

*Generation of B16M-F10 Cells Adapted to a GED—* First we studied the relationship among tumor growth, food intake, and blood Gln levels in B16M-F10-bearing mice fed a standard diet (SD) or a GED (Table 1). As tumor grew locally (after inoculation into the footpad), as compared with non-tumor-bearing controls, food intake decreased significantly in B16M-F10-bearing mice fed an SD or a GED (24 and 21% decrease, respectively, 24 days after tumor inoculation) (Table 1). No significant differences in tumor size were found when the S.D. and the GED group were compared (Table 1). The body weight in both tumor-bearing groups (S.D. and GED) also decreased, as compared with controls (~21 and 7% decrease, respectively, 24 days after tumor inoculation) (Table 1). However, plasma L-Gln levels, which were significantly decreased in B16M-F10-bearing mice fed an SD, were maintained as in control values (non-tumor-bearing) in mice fed a GED (Table 1). In addition, since a critical feature of the host response to a growing cancer is the development of muscle L-Gln depletion (38), skeletal muscle L-Gln release was also measured. As shown in Table 1, skeletal muscle L-Gln release increased more than 100% in B16M-F10-bearing mice fed an SD, whereas that increase was limited to ~20% in B16M-F10-bearing mice fed a GED. Therefore, from the results displayed in Table 1, we can conclude that the GED: (a) does not increase the rate of B16M-F10 growth (as previously suggested in other tumor models, e.g. 18, 23); (b) partially prevents the loss of body weight (whereas the 21% decrease in B16M-F10-bearing mice fed an SD represents a state of cachexia for the host, e.g. Ref. 47); and (c) maintains physiological plasma L-Gln levels, which ensures a higher supply of this amino acid for the tumor, as compared with B16M-F10-bearing mice fed an SD.

The next step forward was to investigate possible GED-induced adaptive alterations in L-Gln metabolism in growing B16M-F10 cells. As shown in Table 2, free L-Gln was not detectable within the tumor cells, which is not surprising, because this amino acid is rapidly metabolized by most growing cancers (e.g. Ref. 48). However, 24 days after inoculation into the footpad, B16M-F10 cells isolated from mice fed a GED showed higher Glnase, glutamate dehydrogenase, alanine transaminase, and aspartate transaminase activities than their equivalents isolated from mice fed an SD; whereas Gln synthetase activity was lower in the GED group (Table 2). These changes in Glnase and Gln synthetase activities correlated with parallel changes in gene expression (Fig. 1). Therefore L-Gln is metabolized faster in B16M-F10 cells isolated from mice fed a GED. As a consequence, as compared with B16M-F10 cells isolated from mice fed an SD diet, L-Glu (Table 2) and other metabolic intermediaries (aspartate, alanine and citrate, not shown) accumulated in mitochondria, and the cytosol of tumor cells was isolated from mice fed a GED. In particular, cytosolic L-Glu (a potential inhibitor of GSH uptake by mitochondria (8, 23)) increased from ~1.7 mM in tumor cells from mice fed an SD to ~3.4 mM in mice fed a GED. Thus L-Glu accumulation in the cytosolic compartment could likely induce a decrease in mtGSH levels in growing B16M-F10 cells adapted to a GED (B16M-F10-Gln− cells). Moreover, our results demonstrate for the first time that it is possible to generate, under in vivo conditions, a highly metabolic state of the tumor cells adapted to a GED.

L-Gln-induced Alterations in the Mitochondrial Glutathione Status of B16M-F10 Cells—Cytosolic and mitochondrial GSH and oxidized glutathione levels were determined in B16M-F10 cells isolated from mice fed an SD or a GED. As shown in Table 3, only mtGSH levels were significantly lower (~52% less) in B16M-F10-Gln− than in B16M-F10 cells. γ-Glutamylcysteine synthetase and GSHs enzyme activities were
found similar in B16M-F10 and B16M-F10-Gln" cells (values were not significantly different of those previously published for B16M-F10 cells (7), not shown), indicating that cytosolic GSH is not affected by the GED. Then we measured GSH uptake by mitochondria isolated from B16M-F10 and B16M-F10-Gln" cells. In agreement with previous observations (7), B16M cell mitochondria showed maximal uptake rates during the first min (Fig. 2). Equilibrium was reached in 1–2 min (Fig. 2) at the external GSH levels calculated for the cytosol of intact cells (see legend to Fig. 2). l-Glu, at the physiological cytosolic concentration measured in B16M-F10-Gln" cells (see above and Table 2), inhibited GSH transport into mitochondria (Fig. 2). Thus, l-Glu-induced inhibition of GSH transport appears involved as the main mechanism responsible of the partial mtGSH depletion found in B16M-F10-Gln" cells. This fact is important, because, as previously demonstrated, the cellular GSH pool relevant for cell survival is the mitochondrial one (7).

TNF-α- and Bcl-2-AS-induced Cytotoxicity in B16M-F10-Gln" Cells—

TNF is a macrophage/monocyte-derived cytokine with cytostatic and cytotoxic anti-tumor effect (49). TNF-α interferes with electron flow in the mitochondria (50) and increases ROS production (24). Addition of sublethal doses of rmTNF-α to cultured B16M-F10 cells induces mtGSH depletion but to a level that is not low enough to promote opening of the PTTPC (7). Nevertheless, in vitro, selective mtGSH depletion in B16M-F10 cells can be obtained (7) by incubation in the presence of l-butihionine (S,R)-sulfoximine (a specific inhibitor of γ-glutamylycysteine synthetase, the rate-limiting step in GSH biosynthesis (51)) and monochlorobimane (a substrate of the GSH transferases originally developed for fluorometric determination of GSH (52)). Under these experimental conditions, the same sublethal doses of rmTNF-α induced a sharp activation of apoptotic death in B16M-F10 cells once mtGSH levels fell below 30% of controls values (5). However buthionine sulfoximine and/or monochlorobimanes or other thiol-depleting agents, such as diethylmaleate (e.g. 5), are not selective for tumor cells and can cause severe systemic toxicity under in vivo conditions (e.g. Refs. 3 and 53). On the other hand, feeding tumor-bearing mice with a GED can induce tumor mtGSH depletion (Table 3) without affecting mtGSH and physiological functions in normal tissues (e.g. Ref. 23 and references therein). Thus the GED approach, which decreases tumor mtGSH to ~50% of control values, may represent a feasible and safe approach to potentiate TNF-α-induced cytotoxicity in highly metastatic cells.

Recently we found that Bcl-2 overexpression, which is a characteristic feature of B16M-10 cells as well as of other malignant tumors (including human tumors of the liver, lung, breast, and ovary (54)), prevented the GSH-depletion associated increase in mitochondrial membrane permeability, although only when mtGSH levels remained above a critical threshold (~30% of control values) (5). However, this critical threshold increased to ~60% of mtGSH control values in Bcl-2-AS-treated B16M-F10 cells (5). Thus combination of a GED, TNF-α, and Bcl-2-AS could be used to challenge survival of B16M cells with high metastatic potential. To test this potential strategy we produced experimental hepatic metastases by intravenous injection (portal vein) of viable B16M-F10 or B16M-F10-Gln" cells (both cell lines were isolated from tumors growing, during 24 days, in the footpads of mice fed an SD or a GED). Ten days after intraportal inoculation, iB16M or iB16M-Gln" cells were isolated from metastatic foci growing in the liver (see under "Experimental Procedures") and cultured. As shown in Fig. 3A, in vitro addition of rmTNF-α to iB16M cells decreased mtGSH (10 h after cytokine addition) to ~71% of control values without significantly affecting cell viability. When rmTNF-α was added to cultured iB16M-Gln" cells, where
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control mtGSH levels are lower than their equivalents in iB16M cells, mtGSH levels further decreased to \(~1.8 \text{ nmol/}10^6\) cells (while iB16M cells contain \(~7.3 \text{ nmol of mtGSH/}10^6\) cells before rmTNF-\(\alpha\) addition) (Fig. 3B). In this second case cell viability decreased by \(~62\%\), as compared with control values, within the 8- to 12-h period after cytokine addition (Fig. 3B). However, when rmTNF-\(\alpha\) was added to cultured Bcl-2-AS-treated iB16M-F10-Gln\(^+\) cells, where mtGSH levels are similar to those found in iB16M-Gln\(^+\) cells, cell viability further decreased (by \(~88\%\), as compared with control values) and much earlier (a sharp decrease in cell viability started 2 h after cytokine addition) (Fig. 3C). Therefore, Bcl-2-AS treatment in L-Gln-adapted metastatic cells can facilitate TNF-\(\alpha\)-induced cytotoxicity even when mtGSH levels are higher than the critical threshold (30% of control values) mentioned above.

Depletion of mtGSH, which is not synthesized by mitochondria but taken up from the cytosol (Ref. 7 and references therein), can cause a fall in mitochondrial membrane potential, opening of the PTPC and the release of proapoptotic molecular signals. Thus we tested, in our experimental conditions, whether Bcl-2-AS and TNF-\(\alpha\) treatment activates the mitochondrial-based molecular mechanism of cell death in iB16M-Gln\(^+\) cells. As shown in Table 4, rmTNF-\(\alpha\)-induced ROS generation associated with mtGSH and mitochondrial ATP depletion, a decrease in mitochondrial membrane potential, and an increase in cytosolic cytochrome \(c\) level and caspase 3 activity. The mitochondrial membrane potential was significantly lower in Bcl-2-AS- and rmTNF-\(\alpha\)-treated iB16M-Gln\(^+\) cells than in rmTNF-\(\alpha\)-treated iB16M-Gln\(^+\) cells (Table 4). Whereas cytochrome \(c\) and caspase 3 were significantly higher in Bcl-2-AS- and rmTNF-\(\alpha\)-treated iB16M-F10-Gln\(^+\) cells than in rmTNF-\(\alpha\)-treated iB16M-Gln\(^+\) cells (Table 4). In addition, to prove that mtGSH is directly involved in this mechanism, we used GSH ester (which readily enters the cell and delivers free GSH, e.g. (26)) and found that mtGSH replenishment prevented the molecular activation of cell death in B16M-F10 cells treated with TNF-\(\alpha\) and/or Bcl-2-AS (Table 4).

Oxidative Stress-resistant Bcl-2-AS-treated B16M-F10-Gln\(^+\) Cells Overexpress Antioxidant Enzyme Activities—Despite the strong activation of death mechanisms in Bcl-2-AS- and rmTNF-\(\alpha\)-treated iB16M-Gln\(^+\) cells, cell number and viability only decreased to \(~34\%\) and \(~75\%\), respectively, of control values (untreated iB16M-Gln\(^+\) cells) (see below). Thus it is plausible that rmTNF-\(\alpha\) and/or Bcl-2-AS treatment could activate programs of defense in iB16M-Gln\(^+\) cells leading to higher resistance in the surviving cell subset. Because rmTNF-\(\alpha\)-treatment increased ROS generation (Table 4), we investigated as a first option the effect of Bcl-2-AS and rmTNF-\(\alpha\) on antioxidant enzyme activities. As shown in Table 5, Bcl-2-AS treatment did not affect the antioxidant enzyme system in iB16M-Gln\(^+\) cells. However, addition of rmTNF-\(\alpha\), as compared with untreated cells, caused approximately a 5-fold increase in Mn-SOD activity and a 2-fold increase in CAT activity (Table 5). This increase in activities correlated with overexpression of both enzymes (Fig. 4).

Mn-SOD Silencing Abrogates Resistance to TNF-\(\alpha\) in Bcl-2-AS-treated B16M-F10-Gln\(^+\) Cells—As indicated above, the rmTNF-\(\alpha\)-dependent increase in Mn-SOD and CAT activities was quantitatively more important in the first case. Moreover, interestingly, Huang et al. (55) suggested that malignant cells may be highly dependent on SOD for survival and proposed SOD activity as a possible target for the selective killing of cancer cells. Based on this suggestion and on our present results we investigated whether a combination of two different antisense oligonucleotides, Bcl-2-AS and SOD2-AS could prevent resistance of iB16M-Gln\(^+\) cells against rmTNF-\(\alpha\)-induced oxidative stress. As shown in
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**TABLE 4**

ROS generation and the molecular activation of apoptosis upon rmTNF-α administration to iB16M-Gln+ and Bcl-2-AS-treated iB16M-Gln+ cells

rmTNF-α and Bcl-2-AS were added to the culture medium as indicated in the caption to Fig. 3. GSH ester (0.5 mM) was added 2 h before addition of the cytokine. Measurements were performed 8 h after rmTNF-α addition in iB16M-Gln+ cells and 2 h after rmTNF-α addition in Bcl-2-AS-treated iB16M-Gln+ cells (see Fig. 3). Bcl-2 levels in Bcl-2-AS-treated iB16M-Gln+ cells (see the legend of Fig. 3) were not altered significantly by addition of rmTNF-α or GSH ester. Data are means ± S.D. of 5 or 6 different experiments.

| Parameters | Untreated controls | +rmTNF-α | +rmTNF-α+GSH ester | +rmTNF-α | +rmTNF-α+GSH ester |
|------------|--------------------|----------|---------------------|----------|---------------------|
| H2O2 (nmol/106 cells × min) | 0.53 ± 0.14 | 2.10 ± 0.36<sup>a</sup> | 0.79 ± 0.17<sup>a</sup> | 0.60 ± 0.15 | 2.34 ± 0.44<sup>a</sup> |
| O2 (ΔF1) | 2.35 ± 0.47 | 4.64 ± 0.52<sup>a</sup> | 2.77 ± 0.36 | 2.57 ± 0.36 | 4.45 ± 0.61<sup>a</sup> |
| mtGSH (nmol/106 cells) | 3.5 ± 0.7 | 1.5 ± 0.4<sup>a</sup> | 6.9 ± 1.1<sup>a</sup> | 3.3 ± 0.5 | 1.5 ± 0.3<sup>a</sup> |
| MMP (TPM accumulation ratio, %) | 90 ± 7 | 55 ± 8<sup>a</sup> | 98 ± 2 | 70 ± 9<sup>a</sup> | 30 ± 6<sup>a</sup> |
| mtATP (nmol/106 cells) | 1.02 ± 0.30 | 0.60 ± 0.21 | 1.07 ± 0.23 | 0.75 ± 0.16 | 0.41 ± 0.12<sup>a</sup> |
| Cytoplasmic cytochrome c (% of control) | 100 ± 15 | 180 ± 26<sup>a</sup> | 120 ± 19 | 157 ± 20<sup>a</sup> | 210 ± 35<sup>a</sup> |
| Caspase 3 (pnmol/106 cells × min) | 1.45 ± 0.25 | 3.43 ± 0.60<sup>a</sup> | 1.82 ± 0.17<sup>a</sup> | 2.06 ± 0.39<sup>a</sup> | 3.91 ± 0.78<sup>a</sup> |

Means ± S.D.

<sup>a</sup>p < 0.01.

<sup>b</sup>p < 0.05 comparing all conditions under Bcl-2-AS-treated iB16M-Gln+ cells versus untreated controls.

**TABLE 5**

Antioxidant enzyme activities in Bcl-2-AS-treated iB16M-Gln+ and rmTNF-α-resistant Bcl-2-AS-treated iB16M-Gln+ cells

Enzyme activities were measured in viable iB16M-Gln+ cell variants as indicated under “Experimental Procedures.” Tumor cells were treated with Bcl-2-AS- and/or rmTNF-α as indicated in the caption to Fig. 3, and then viable survivors were cultured again for 60 h before measurement of enzyme activities. CuZn-SOD and Mn-SOD activities are expressed as units/10<sup>6</sup> cells, whereas CAT, glutathione peroxidase, glutathione reductase, and GAPDH are expressed as milliunits/10<sup>6</sup> cells. Data are means ± S.D. of four or five different experiments.

| Enzyme | iB16M cell variants | iB16M-Gln+ | Bcl-2-AS-treated iB16M-Gln+ | Bcl-2-AS- and rmTNF-α-treated iB16M-Gln+ |
|--------|---------------------|------------|----------------------------|------------------------------------------|
| CuZn-SOD (units/10<sup>6</sup> cells) | 1.5 ± 0.3 | 1.6 ± 0.4 | 1.6 ± 0.3 |
| Mn-SOD (units/10<sup>6</sup> cells) | 0.2 ± 0.05 | 0.3 ± 0.1 | 1.0 ± 0.2<sup>a</sup> |
| CAT (milliunits/10<sup>6</sup> cells) | 4.1 ± 0.6 | 4.5 ± 0.5 | 9.6 ± 1.0<sup>a</sup> |
| Glutathione peroxidase (milliunits/10<sup>6</sup> cells) | 7.5 ± 1.3 | 7.8 ± 1.5 | 8.1 ± 1.1 |
| Glutathione reductase (milliunits/10<sup>6</sup> cells) | 10.6 ± 1.7 | 9.7 ± 1.9 | 11.2 ± 2.3 |
| GAPDH (milliunits/10<sup>6</sup> cells) | 27.4 ± 5.2 | 30.2 ± 7.4 | 29.5 ± 5.6 |

Means ± S.D.

<sup>a</sup>p < 0.01 comparing Bcl-2-AS-treated or Bcl-2-AS- and rmTNF-α-treated iB16M-Gln+ cells versus iB16M-Gln+ cells.

<sup>b</sup>p < 0.01 comparing Bcl-2-AS- and rmTNF-α-treated iB16M-Gln+ cells versus Bcl-2-AS-treated iB16M-Gln+ cells.

**FIGURE 4.** Manganese superoxide dismutase and catalase expression in Bcl-2-AS-treated iB16M-Gln+ and rmTNF-α-resistant Bcl-2-AS-treated iB16M-Gln+ cells.

Northern blot of total cellular RNA (10 μg). From left to right: lane 1, Bcl-2-AS-treated iB16M-Gln+ cells cultured for 24 h; lane 2, viable Bcl-2-AS-treated iB16M-Gln+ cells 12 h after addition of rmTNF-α (see the caption to Fig. 3); lane 3, viable Bcl-2-AS-treated iB16M-Gln+ cells 24 h after addition of rmTNF-α.

Table 6, where growth and type of death was studied in cultured iB16M-Gln+ cells, the combined antisense therapy increased rmTNF-α-induced cytotoxicity (cell number and viability decreased to ~3 and 31%, respectively, of control values for untreated iB16M-Gln+ cells). Therefore, this experimental strategy is able to decrease the number of fully viable malignant cells to ~1% of controls. Mn-SOD activity in SOD2-AS-treated iB16M-Gln+ cells was practically undetectable (see the caption to Table 6). Mn-SOD expression quantitated by RT-PCR analysis was of 1.2 ± 0.2 in control iB16M-Gln+ cells and of 0.02 ± 0.01 in Mn-SOD-AS-treated iB16M-Gln+ cells (n = 4–5; p < 0.01). Only when invasive cells were treated with Bcl-2-AS, SOD2-AS and rmTNF-α was necrotic cell death preferential (Table 6), likely indicating in this case a switch from the mitochondrion-dependent apoptotic cell death activation to a cellular bioenergetic catastrophe (56).

As a consequence of Mn-SOD-AS-induced changes in Mn-SOD and CAT activities (see the caption in Table 6), generation of H2O2 and O2 should also be affected. Generation of H2O2 in Bcl-2-AS plus Mn-SOD-AS-treated and in Bcl-2-AS plus Mn-SOD plus rmTNF-α-treated iB16M-Gln+ cells was of 0.30 ± 0.08 and 0.26 ± 0.06 nmol/106 cells × min (n = 4–5 in each case); which represents in both cases about a 50% decrease as compared with untreated controls, and about a 10% of the value obtained in rmTNF-α-treated iB16M-Gln+ cells (see the data reported in Table 4). On the other hand, generation of O2 in Bcl-2-AS plus Mn-SOD-AS-treated and in Bcl-2-AS plus Mn-SOD-AS plus rmTNF-α-treated iB16M-Gln+ cells was of 3.17 ± 0.38 and 9.42 ± 0.86 (ΔF1) (n = 4–5 in each case), which in the second case represents an increase of ~2-fold increase in O2 generation as compared with the value found (Table 4) in Bcl-2-AS plus rmTNF-α-treated iB16M-Gln+ cells. Therefore Mn-SOD-AS-induced high levels of O2 must be directly involved in the mechanism leading to the huge number of deaths found in Bcl-2-AS plus Mn-SOD-AS plus rmTNF-α-treated iB16M-Gln+ cells (Table 6).
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**TABLE 6**

Antisense manganese superoxide dismutase oligodeoxynucleotides abrogate resistance of Bcl-2-AS-treated iB16M-Gln<sup>+</sup> cells to TNF-α-induced cytotoxicity

| Parameters | iB16M-Gln<sup>+</sup> | + Bcl-2-AS | + Bcl-2-AS + Mn-SOD-AS |
|------------|-----------------------|------------|------------------------|
|            | No antisense treatment | + rmTNF-α | + rmTNF-α | + rmTNF-α |
| Cell number (10<sup>6</sup> cells) | 2.25 ± 0.34 | 2.08 ± 0.27 | 1.92 ± 0.25 | 1.92 ± 0.25 |
| Cell viability (%) | 98.2 ± 1.3 | 97.5 ± 1.0 | 90.4 ± 3.7<sup>a</sup> | 31.0 ± 4.8<sup>b</sup> |
| Necrotic cells (%) | 45 ± 4 | 39 ± 6 | 42 ± 7 | 42 ± 7 |
| Apoptotic cells (%) | 55 ± 7 | 61 ± 6 | 58 ± 6 | 58 ± 6 |

Note: Data are means ± S.D. of 6 or 7 different experiments.

**DISCUSSION**

TNF-α can cause hemorrhagic necrosis and regression of experimental tumors and is being utilized as an antineoplastic agent for the treatment of patients with locally advanced solid tumors (49, 59–61). Although paradoxically some pre-clinical findings suggest that pathophysiological concentrations of endogenous TNF-α can promote tumor genesis and growth, high pharmacological doses of TNF-α combined with chemotherapy may help to regress otherwise intractable tumors (60). Thus, efforts continue to optimize delivery to avoid severe toxicities. Indeed, TNF-α doses required to cure tumor-bearing mice lead to injury of normal tissues and, eventually, may cause a lethal shock syndrome (62–63). This toxicity implies drastic limitations for the therapeutic use of TNF-α. In the medical practice high dose TNF-α plus chemotherapy, with or without IFN-γ, can be administered regionally through isolated limb perfusion. This procedure may produce, e.g., between 70 and 80% complete remission in cases of in transit melanoma with chemotherapy may help to regress otherwise intractable tumors (60). Thus, efforts continue to optimize delivery to avoid severe toxicities. Indeed, TNF-α doses required to cure tumor-bearing mice lead to injury of normal tissues and, eventually, may cause a lethal shock syndrome (62–63). This toxicity implies drastic limitations for the therapeutic use of TNF-α. In the medical practice high dose TNF-α plus chemotherapy, with or without IFN-γ, can be administered regionally through isolated limb perfusion. This procedure may produce, e.g., between 70 and 80% complete remission in cases of in transit melanoma...
metastases and between 25 and 36% complete remission in cases of inexcitable soft-tissue sarcomas. This combination of cytokines and drugs represents a dual targeting therapy causing apoptosis of angiogenic endothelium and tumor cells (64). Thus, approaches that could potentiate the effect of TNF-α in vivo, without causing severe toxicity, would be beneficial for the treatment of systemically disseminated malignant tumors.

The deleterious effect of free radicals on mitochondria is largely due to the opening of a cyclosporin A-sensitive and Ca2+-dependent transmembrane permeability transition pore (e.g. Refs. 56 and 65). A current hypothetical version of this model proposes that Ca2+ triggers a conformational change affecting, at least, the voltage-dependent anion channel and the adenine nucleotide translocase, which would form a non-specific channel. The process is greatly facilitated by the binding of cyclophilin D and by oxidation of thiol groups on the adenine nucleotide translocase (56, 66). Mitochondrial GSH is the only defense against peroxides generated from the electron transfer chain (67). Depletion of the mtGSH pool causes a major increase in TNF-α-induced free radicals and, thereby, mitochondrial dysfunction and tumor cell damage (7, 10, 45) (Fig. 3, Table 4). L-Gln-enriched nutrition can cause selective mtGSH depletion in tumor cells (10) and thus may facilitate TNF-α-induced lipid peroxidation, loss of mitochondrial functions, PTPC opening, and the release of proapoptotic molecular signals (see Fig. 3 and Table 4). In the present report we developed an original methodology to produce L-Gln-adapted malignant cells with very high metastatic potential (Tables 1 and 2, and Fig. 3). B16M-F10-Gln− cells grew as a local tumor in the footpad of mice fed a GED which, as compared with control tumor-bearing mice fed an SD diet, did not accelerate cancer growth (Table 1). Moreover, the GED significantly decreased the loss of body weight and the release of skeletal muscle L-Gln (Table 1), which suggests anticaemic properties. Indeed, in randomized, double-blind controlled clinical trials, cancer patients receiving L-Gln-supplemented parenteral nutrition had improved nitrogen balance, a diminished incidence of clinical infections and less extracellular fluid accumulation, clinical facts that are consistent with the potential role of L-Gln in stimulating protein synthesis in skeletal muscle, supporting endothelial function and integrity, and enhancing immune functions (see Refs. 14 and 20 for a review). Furthermore, in rats, provision of a GED during whole abdominal irradiation (10 Gy, a dose that results in a 50% mortality rate in a few days) exerted a protective effect on the small bowel mucosa by supporting crypt cell proliferation and, thereby, accelerated healing of the irradiated bowel and reached a 100% survival of the irradiated animals (18, 20). Besides, L-Gln supplementation may also decrease the incidence and/or severity of chemotherapy-associated mucositis, irinotecan-associated diarrhea, paclitaxel-induced neutropathy, hepatic veno-occlusive disease in the setting of high dose chemotherapy and stem cell transplantation, and the cardiotoxicity that accompanies anthracycline use (20). Studies in patients receiving L-Gln-enriched nutrition for several weeks confirmed the clinical safety of this approach in a catabolic patient population (68). In conclusion, supplementation of a GED appears a feasible methodology to deplete mtGSH levels (Table 3) in growing malignant cells, and it may promote beneficial effects for the cancer patient.

Highly metastatic B16M-F10 cells, as compared with the low metastatic B16M-F1 line, overexpress preferentially Bcl-2 (5). The proto-oncogene Bcl-2 and its anti-apoptotic homologs are mitochondrial membrane permeabilization inhibitors (69) and participate in development of chemoresistance (70), whereas expression of pro-death genes, e.g. Bax or Bak, is often reduced in cancer cells (71). The importance of GSH in modulating the ability of Bcl-2 to prevent apoptosis was first detected in GT1–7 neurons where Bcl-2-induced suppression of apoptosis required maintenance of physiological GSH levels (72). Recently, we observed that death in B16M-F10 cells was sharply activated in vitro at mtGSH levels below 30% of control values; although this critical threshold increased to ~60% of control values in Bcl-2-AS-treated cells (5). Here we have shown that adaptations to a GED (Table 2) facilitates mtGSH depletion in malignant cells under in vivo conditions (Table 3 and Fig. 2). When iB16M-F10-Gln− cells were treated with a combination of Bcl-2-AS and rmTNF-α, their viability decreased to ~12% of control values (see under “Results”). This small percentage of Bcl-2-AS- and rmTNF-α-resistant invasive cells follow further adaptations. We found that Mn-SOD and CAT activities were overexpressed in these survivors (Table 5 and Fig. 4). Mn-SOD is particularly interesting, because numerous in vivo studies show that the SODs can be highly expressed in aggressive human tumors and that high SOD activities have been associated with poor prognosis and with resistance to cytotoxic drugs and radiation (see Ref. 73 for a review). Paradoxically, increased Mn-SOD expression has been also correlated in different cancer cell types (including human melanoma cells, e.g. (74)) with suppression of neoplastic transformation, decreased metastatic potential, decreased proliferation in vitro, and reversion of malignant phenotype (73). However, transgenic mice overexpressing both Mn-SOD and glutathione peroxidase (scavenger of H2O2) were found to have an increased risk for skin carcinogenesis compared with controls (75); and, besides, Mn-SOD-overexpressing cheek pouch carcinoma cells have shown increased in vitro invasiveness, which was partially inhibited by co-overexpression of Mn-SOD and CAT (76) (see also Fig. 4). These controversial facts indicate the importance of (a) the balance of ROS-mediated effects/signals in cells, and (b) the net redox state, which has never been assessed accurately in malignant cells. Furthermore it is important to point out that iB16M-F10-Gln− cells surviving after treatment with Bcl-2-AS and rmTNF-α may undergo, theoretically, many other adaptations. For instance, in mammalian cells at least 40 different gene products are involved in adaptive responses to oxidative stress (77). Despite these potential multigene expression adaptations, we found that by including SOD2-AS in our combined treatment the percentage of survivors decreased to ~1% (Table 6). These results are in agreement with previous observations showing that when, e.g., squamous cell carcinomas were transplanted with Mn-SOD-AS and inoculated subcutaneously into mice, the cancer cells grew slower and these mice lived longer than their controls (78).

Bcl-2 antisense therapy is feasible, because it has been shown, e.g. using G3139, an 18-base phosphorothioate oligonucleotide complementary to the first six codons of the Bcl-2 mRNA, which selectively and specifically inhibits Bcl-2 expression and promotes apoptosis in different human and murine cancer cell lines (79). Besides, in B16M-F10 cells, acceleration of GSH efflux and the consequent GSH depletion can be achieved by using Bcl-2-AS (which avoids the Bcl-2-induced inhibition of GSH efflux through the cystic fibrosis transmembrane conductance regulator), verapamil (a multidrug resistance protein 1 activator that facilitates GSH efflux through this channel, and acivicin-induced inhibition of γ-glutamyl transpeptidase (which limits GSH synthesis by preventing cysteine generation from extracellular GSH) (6). These mechanisms, by depleting first cytosolic GSH, would further limit GSH availability for mitochondrial uptake. On the other hand, in agreement with SOD2 knock-out studies (80), decreased Mn-SOD activity achieved by antisense oligonucleotides leads to increased sensitivity to variable stress conditions in vitro (e.g. TNF-α, interferon-γ, radiation, hyperthermia, etc.) (73). Moreover, as recently reported, in vivo SOD2 antisense treatment is also feasible (41); our experimental approach can
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be easily combined with chemotherapy and/or ionizing radiations to further improve its efficacy and to prevent survival of some cells under dormancy conditions (81, 82). Indeed, as shown under "Results" a combination of taxol and daunorubicin completely eliminated the last hr-iB16M-Gln− survivors. Naturally, although the situation in vivo can be much more complex due to multiple interactions of tumor cells with their microenvironment, it is also important to emphasize that in our in vitro experiments some physiological anti-tumor defenses (e.g., the cytotoxic action elicited by the immune system and the vascular endothelium) were absent.

In conclusion, the novel facts reported in this report are: (a) supplementation of a GED deplets mtGSH levels in highly metastatic B16M-F10 cells growing in vivo; (b) combined GED-induced mtGSH depletion and Bcl-2-AS-induced Bcl-2 depletion sensitizes invasive B16M-F10 cells to TNF-α; (c) oxidative stress-resistant metastatic cell survivors show an adaptive response that includes overexpression of Mn-SOD; (d) treatment of Bcl-2-depleted/TNF-α-resistant iB16M-Gln+ cells with Mn-SOD-AS further decreases metastatic cell viability to only 1% as compared with untreated control values; and (e) chemotherapy, which is useless if administered in a previous step, can then eliminate this final metastatic cell subset. The biochemical mechanisms described in this report identify critical molecules that can be sequentially targeted (see Fig. 5) to facilitate elimination of highly resistant metastatic cells. Thus, application of our strategy may possibly help to improve the poor prognosis of patients bearing a highly malignant melanoma (and likely other malignant tumors with common molecular characteristics).

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