Acceleration of Glutathione Efflux and Inhibition of γ-Glutamyltranspeptidase Sensitize Metastatic B16 Melanoma Cells to Endothelium-induced Cytotoxicity*

Highly metastatic B16 melanoma (B16M)-F10 cells, as compared with the low metastatic B16M-F1 line, have higher GSH content and preferentially overexpress BCL-2. In addition to its anti-apoptotic properties, BCL-2 inhibits efflux of GSH from B16M-F10 cells and thereby may facilitate metastatic cell resistance against endothelium-induced oxidative/nitrosative stress. Thus, we investigated in B16M-F10 cells which molecular mechanisms channel GSH release and whether their modulation may influence metastatic activity. GSH efflux was abolished in multidrug resistance protein 1 knock-out (MRP−/−) B16M-F10 transfected with the Bcl-2 gene or in MRP−/− B16M-F10 cells incubated with L-methionine, which indicates that GSH release from B16M-F10 cells is channeled through MRP1 and a BCL-2-dependent system (likely related to an L-methionine-sensitive GSH carrier previously detected in hepatocytes). The BCL-2-dependent system was identified as the cystic fibrosis transmembrane conductance regulator, since monoclonal antibodies against this ion channel or H-89 (a protein kinase A-selective inhibitor)-induced inhibition of cystic fibrosis transmembrane conductance regulator gene expression completely blocked the BCL-2-sensitive GSH release. By using a perfusion system that mimics in vivo conditions, we found that GSH depletion in metastatic cells can be achieved by using Bcl-2 antisense oligodeoxynucleotide- and verapamil (an MRP1 activator)-induced acceleration of GSH efflux, in combination with acivicin-induced inhibition of γ-glutamyltranspeptidase (which limits GSH synthesis by preventing cysteine generation from extracellular GSH). When applied under in vivo conditions, this strategy increased tumor cytotoxicity (up to ~90%) during B16M-F10 cell adhesion to the hepatic sinusoidal endothelium.

B16 melanoma (B16M)1 cells with high glutathione (GSH; γ-glutamylcysteinylglycine) content show higher metastatic activity in the liver than those with low GSH content (1). The liver is a common site for metastasis development, and we demonstrated that GSH protects B16M cells against nitrosative and oxidative stress in the murine hepatic microvasculature (2, 3). The concept that high GSH content status is an important factor for metastasis progression was strongly supported by the fact that metastatic B16M cell survival and growth can be enhanced by directly increasing their GSH content with GSH ester, which readily enters the cell and delivers free GSH (4, 5). In consequence, the maintenance of high intracellular levels of GSH appears critical for metastatic cells to survive intravascularly and to progress extravascularly.

Multidrug and/or radiation resistance, which are characteristic features of malignant tumors, frequently associate with high GSH content in the cancer cells (6). Efflux of GSH and GSH S-conjugates from different mammalian cells is mediated by multidrug resistance proteins (MRP), among which MRP1 and MRP2 have been characterized at the functional level as ATP-dependent pumps with broad specificity for GSH and glucuronic or sulfate conjugates (7–10). Multidrug resistance frequently associates with the overexpression of P-glycoprotein and/or MRP1 (11), both functioning as pumps that extrude drugs from tumor cells. GSH depletion induced by L-buthionine-(SR)-sulfoximine (BSO), a specific inhibitor of γ-glutamylcysteine synthetase (the rate-limiting step in GSH biosynthesis) (12), resulted in a complete reversal of resistance to anticancer drugs of different cell lines overexpressing MRP1 but had no effect on P-glycoprotein-mediated multidrug resistance (13). Most interestingly, cancer cells can release GSH through MRP1 even in the absence of cytotoxic drugs (7).

In a recent study we demonstrated that B16M-F10 cells with a high metastatic potential overexpress BCL-2, show an increase in intracellular GSH content, show no change in the GSH synthesis rate, but show a decrease in GSH efflux (5). This study also provides evidence that BCL-2 can directly inhibit GSH export, thereby accounting for the increase in intracellular GSH. Moreover, it demonstrates that GSH depletion and BCL-2 antisense therapy can sensitize cells to TNF-

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α-induced apoptotic death. In consequence, it is plausible that BCL-2, in addition of its anti-apoptotic properties (14), may also increase metastatic cell resistance against oxidative/nitrosative stress by preserving intracellular GSH. In fact, GSH efflux prior or during apoptosis appears an essential regulator of the apoptotic killing mechanism (15).

Because GSH efflux from B16M cells can be increased by using Bcl-2 antisense oligodeoxynucleotides (Bcl-2-AS) (5), and possibly by using different MRPs regulators, the aim of the present report was to investigate whether the rate of efflux may become an important factor regulating intracellular GSH content and thereby metastatic cell survival.

**EXPERIMENTAL PROCEDURES**

**Culture of B16M F10 Cells**—Murine B16M F10 cells (from the ATCC, Manassas, VA) were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen), pH 7.4, supplemented with 10% fetal calf serum (Invitrogen), 10 mM HEPES, 40 mM NaHCO3, 100 units/ml penicillin, and 100 μg/ml streptomycin (4). Cell integrity was assessed by trypan blue exclusion and leakage of lactate dehydrogenase activity (4). GSH and GSSG—GSH was measured by the glutathione-S-transferase system (Clontech) as described previously (17). Total glutathione (GSH + 2GSSG) was determined by a kinetic assay in which a catalytic amount of GSH or GSSG and glutathione reductase cause the continuous reduction of 5,5′-dithio-bis-2-nitrobenzoic acid (Sigma) by NADPH (16). GSH monoisopropyl(glycyl) ester was prepared as described previously (17).

**Bcl-2 Gene Transfer and BCL-2 Analysis—**B16M cells were transfected with 1,000 nontransfected B16M cells).

**Based assay from Sigma (1 unit of BCL-2 was defined as the amount of**

**fraction by enzyme immunoassay (18) using a monoclonal antibody-in-**

**instructions. BCL-2 protein was quantitated in the soluble cytosolic**

**Bcl-2 protein levels were expressed as arbitrary fluorescence units (**

**pared as described previously (17).**

**Bcl-2 Gene Transfer and BCL-2 Analysis—**The Tet-Off gene expression system (Clontech) was used, as in Ref. 5, to insert the mouse Bcl-2 gene and for transfection into B16M cells following the manufacturer’s instructions. BCL-2 protein was quantitated in the soluble cytosolic fraction by enzyme immunoassay (18) using a monoclonal antibody-based assay from Sigma (1 unit of BCL-2 was defined as the amount of BCL-2 protein in 1,000 nontransfected B16M cells).

**Cellular BCL-2 protein levels were also analyzed, as recently reported (5).**

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**Northern Blot Analysis—**Total RNA was isolated using the TRIzol kit from Invitrogen and following the manufacturer’s instructions. Ten μg of total RNA were electrophoresed in 1% agarose gels containing formaldehyde. RNA was electrophoretically transferred to a nylon membrane (Hybond-N, Amersham) and cross-linked by ultraviolet light. Membrane-bound UV cross-linking. Murine CFTR and control glyceraldehyde-3-phosphate dehydrogenase (G3PDH, Clontech) cDNA probes were radiolabeled with 32P by random priming (23). The membranes were hybridized at 65°C with the 32P-labeled cDNA fragments. After washing at room temperature, the filters were exposed to film, and autoradiographs were photographed using a PhosphorImager (Bio-Rad).

**Reverse Transcription-PCR—**A 392-bp region corresponding to nucleotides 1340–1730 of the mouse CFTR gene (22) was amplified with the forward primer 5′-GGAGGGAGGAGTTGAGGAA-3′ and the reverse primer 5′-GTGATGTCGCTGGATGTTG-3′. The CFTR 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 containing the specific primers and AmpliTaq Gold DNA polymerase (Applied Biosystems) was then added. Amplifications were performed in a GeneAmp 2400 thermal cycler (PerkinElmer Life Sciences).

**Quantitative Determination of the Plasma Membrane Potential—**Plasma membrane potential (PMP) was measured using a standard technique (21). B16M-F10 cells were cultured for 24 h, as described above, but seeded at 5 × 104 cells/cm2 in glass Petri dishes. Culture dishes were filled with 3M KCl and with a 20-megohm DC resistance. Membrane potentials were measured with a WP Instruments M4-A electrometer amplifier (Sarasota, FL), and the output was displayed using a MacLab System (Castle Hill, Australia). Measurements were made only in cells (from at least four different preparations) that gave a stable membrane potential within 10 s of penetration, which indicates a good seal of the plasma membrane with the recording electrode.

**Amino Acid Analysis—**Amino acid analysis was performed using standard methods (29).

**Accumulation Verapamil and Acivicin Cellular Pharmacokinetics—**Accumulation of 10 μM verapamil (at 2 ml/min) was constant throughout the experiment. Efflux flow was monitored continuously for O2 and pH with Philips electrodes. Tumor cell viability was always >97% along the experimental time. A syringe was introduced into the chamber through a rubber septum to take samples (0.5 ml) of the cell suspension without interrupting the flow.

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Cells were centrifuged and washed twice with 1 ml of ice-cold PBS. After centrifugation, cell pellets were solubilized in 1% SDS, and cell-associated radioactivity was determined by liquid scintillation counting.

GH-related Enzyme Activities—B16M-F10 cells were detached (see above), washed twice at 4 °C in Krebs-Henseleit bicarbonate medium (without Ca2+ or Mg2+) containing 0.5 mM EGTA, pH 7.4, resuspended, and homogenized in 0.1 M phosphate buffer, pH 7.2, at 4 °C. γ-Glutamylcysteine synthetase (γ-GCS) and GSH synthetase activities were measured as described elsewhere (4).

Measurement of H2O2—The assay of H2O2 production was based 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) that is mediated by horseradish peroxidase.

Cystine Uptake—B16M-F10 cells were plated in 25-cm2 culture dishes. At the required times, cells were rinsed three times with prewarmed transport medium (10 mM PBS, pH 7.4, with 0.01% CaCl2, 0.01% MgCl2 and 0.1% glucose). Uptake measurement was initiated by addition of 0.1 ml of transport medium containing 1 μCi of [3H]cystine (Amersham Biosciences) and nonradioactive l-cystine (0.5 mM). After incubation at 37 °C, uptake was initiated by rinsing several times with ice-cold PBS until less than 0.001% of the initial radioactivity was measured as described elsewhere (4).

γ-Glutamyltranspeptidase Assay—γ-GT activity was measured as described previously (30). Protein was determined with the BCA protein assay (Pierce). Data were analyzed by one- or two-way ANOVA or t tests where appropriate (SPSS 9.0 software for Windows; SPSS Inc., Chicago). The homogeneity of the variances was analyzed by the Levene test. The null hypothesis was accepted for all the values of the tests in which the F value was nonsignificant at p < 0.05. The data for which the F value was significant was examined by Tukey’s test at p < 0.05.

RESULTS

MRP1 and a BCL-2-dependent System Channel GSH Efflux from B16M-F10 Cells—Recently, we reported that GSH efflux was significantly reduced in B16M-F10 cells (5). Moreover, GSH release increased in B16M-F10/Tet-BCL-2 cells loaded with anti-BCL-2 antibodies (5), thus suggesting that BCL-2 directly inhibits the transport system.

We investigated further the mechanisms through which GSH is released from highly metastatic cells. As shown in Table I, GSH efflux from B16M-F10 cells was decreased by l-methionine suggesting that, in part, GSH is released through a system possibly similar to an L-methionine-sensitive sinusoidal GSH transporter detected in hepatocytes (31, 32). However, GSH efflux was not inhibited by l-methionine in B16M-F10/Tet-BCL-2 (Table I), suggesting that BCL-2 and l-methionine act on the same transport system (similar results were reported previously in HeLa cells (33)). Further experiments using MRP—/— or MRP—/—/Tet-BCL-2 clones showed that GSH efflux was decreased, as compared with controls, in MRP—/— B16M-F10 and in B16M-F10/Tet-BCL-2 cells (Table I). GSH efflux in MRP—/— B16M-F10/Tet-BCL-2 cells was practically abolished, and identical results were obtained in MRP—/— B16M-F10 cells incubated in the presence of methionine (Table I).
**Table 1**

| B16M-F10 | GSH efflux | Tet-BCL-2 | GSH efflux |
|-----------|------------|-----------|------------|
| None      | 32 ± 4 \(^a\) | 1.8 ± 0.3\(^a\) | 45 ± 5\(^p\) | 0.9 ± 0.3\(^p\) |
| t-methionine | 47 ± 3 \(^a\) | 0.7 ± 0.2\(^p\) | 48 ± 3\(^p\) | 0.7 ± 0.3\(^p\) |
| MRP−/− | 45 ± 5\(^p\) | 1.0 ± 0.2\(^p\) | 49 ± 5\(^p\) | 0.1 ± 0.05\(^b\) |
| MRP−/− | 33 ± 4\(^a\) | 1.7 ± 0.2\(^p\) | 46 ± 6\(^p\) | 0.8 ± 0.2\(^p\) |
| t-methionine + MRP−/− | 48 ± 5\(^p\) | 0.1 ± 0.05\(^b\) | 49 ± 4\(^p\) | 0.07 ± 0.03\(^b\) |

Taken together these results indicate that a BCL-2-dependent system and MRPI are the main mechanisms channeling GSH efflux from B16M-F10 cells.

**Molecular Nature of the BCL-2-dependent GSH Channel in B16M-F10 Cells**—BCL-2 overexpression has been associated with plasma membrane hyperpolarization (34), and it was suggested that changes in the rate of GSH efflux could be the consequence of changes in membrane polarity (e.g., Ref. 35).

We explore first this possibility in our experimental model. The PMP measured in B16M-F10, B16M-F10/Tet-BCL-2, and Bel-2-AS-treated B16M-F10 cells cultured for 24 h was 62.3 ± 7.1, 75.4 ± 6.2 (\(p < 0.05\)), and 60.3 ± 5.5 mV, respectively (\(n = 3–4\) in each case). Therefore, BCL-2 overexpression (B16M-F10/Tet-BCL-2) indeed increased significantly the PMP as compared with controls (B16M-F10), which in our model associated with a decrease in GSH efflux (Table I). However, it has been claimed that an increase in GSH efflux is the consequence of hyperpolarization (36). However, we observed that BCL-2 depletion increased GSH efflux from B16M-F10 cells (Table II) without affecting the PMP (see above), and addition to cultured B16M-F10 cells of ouabain (1 \(\mu\)M), a classical inhibitor of the plasma membrane Na\(^+\)/K\(^+\)-ATPase, decreased the PMP to −63% of control values (see above) but did not affect significantly rates of GSH efflux measured in controls, in the presence of t-methionine (1 \(\mu\)M) or in MRP−/− cells (see e.g., Table I). Apparently, efflux of GSH is not driven in B16M-F10 cells by a significant change in the PMP. Our results appear in agreement with those reported by Liu et al. (37) for Hep G2 cells, a human-derived hepatoma cell line, where a fall in the membrane potential produced by the replacement of Na\(^+\) with equivalent K\(^+\) did not affect GSH efflux significantly. Neither ouabain, vanadate (a Ca\(^2+\)/ATPase inhibitor), nor BaCl\(_2\) (a K\(^+\) channel blocker) significantly affected the GSH efflux; however, t-methionine (1 \(\mu\)M) decreased GSH efflux from Hep G2 cells (37).

The CFTR, a member of the ABC family of membrane transport proteins with structural similarities with the MRPI, forms a phosphorylation- and ATP-dependent channel permeable to Cl\(^−\) and to other larger organic anions, including GSH (38). The CFTR is expressed in different cell types (39), and thus we also explored this possibility. GSH efflux increased significantly in Bel-2-AS-treated B16M-F10 cells (containing −3% of control BCL-2 levels, see the legend to Fig. 1), whereas GSH efflux decreased to −50% of control values in MRP−/− B16M-F10 cells (Fig. 1). The difference represents the BCL-2-dependent GSH efflux (see also data in Table I). Monoclonal antibodies anti-CFTR practically abolished the BCL-2-dependent GSH efflux from B16M-F10 cells (Fig. 1; the presence of CFTR in B16M-F10 cells was confirmed with a Western blot, not shown). In consequence, addition of anti-CFTR antibodies to Bel-2-AS-treated MRP−/− B16M-F10 cells practically abolished GSH efflux (Fig. 1).

CFTR is activated by the binding of ATP to its cytoplasmic nucleotide-binding domain and by phosphorylation of key serine residues in the regulatory domain. Phosphorylation is mediated principally by cAMP-dependent protein kinase A (PKA) and by protein kinase C (although to a less degree than the activation by PKA) (40). Basal expression of the CFTR gene is dependent on PKA activity (41). Treatment of human colon carcinoma T84 cells with the PKA-selective inhibitor N\(^\prime\)-[\(\beta\)-bromocinnamylaminoethyl]-5-isouquinolinesulfonamide (H-89) (Seikagaku America, Rockville, MD) caused a complete suppression of CFTR gene expression without affecting other constitutively active genes (41). Thus, we also used this approach in our experimental model. As shown in Fig. 2 treatment with H-89 inhibited CFTR expression in B16M-F10 and in MRP−/− B16M-F10 cells. For the conditions used in Fig. 2, rates of GSH efflux (nmol/10\(^6\) cells \(\times h\)) from wild-type B16M-F10 cells were 1.7 ± 0.2 (similar to control values in Fig. 1); from MRP−/− B16M-F10 cells were 0.8 ± 0.2 (similar to its equivalent value in Fig. 1); from H-89-treated B16M-F10 cells were 1.0 ± 0.15 (similar to the value found in the presence of anti-CFTR, Fig. 1); and from H-89-treated MRP−/− B16M-F10 cells were 0.04 ± 0.02 (which corresponds approximately to a 98% decrease in the rate of efflux) (\(n = 4\) for each condition; and \(p < 0.01\) in all cases, as compared with control wild-type B16M-F10 cells).

These results indicate that CFTR is directly involved in channeling GSH from the cytoplasm of B16M-F10 cells to the extracellular space and that this mechanism corresponds to the BCL-2-sensitive channel. Nevertheless we cannot rule out the possibility that other mechanism(s) could also be working in other cell types or that different CFTR gene mutations could be found when comparing different cancer cells. These interesting questions, although far beyond the aim of the present report, deserve further investigation.

**Antisense Bel-2 Oligodeoxynucleotides and Verapamil Accelerate GSH Release from B16M-F10 Cells**—Parallel to the BCL-2-sensitive CFTR, two mechanisms of transport of GSH by MRPI have been suggested as follows: passive permeability and a VRP-dependent active transport (42). VRP, an inhibitor of P-glycoprotein-mediated drug efflux, is not transported by MRPI (43) but may also inhibit MRPI-mediated drug extrusion (44). We tested VRP in combination with Bel-2-AS treatment to potentiate GSH efflux from B16M-F10 cells. A perfusion chamber, containing a suspension of B16M-F10 cells, was used as an experimental setup that mimics in vivo conditions by providing a constant supply of glucose, amino acids, and
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B16M-F10 cells were cultured for 3 days in the presence or in the absence of 50 μM Bcl-2-AS or a 2-base mismatch oligodeoxynucleotide (see under "Experimental Procedures"). Then the cells were harvested, cultured again for 24 h in the absence of oligodeoxynucleotides, and used for perfusion experiments. BCL-2 levels in control (none under the heading Additions), Bcl-2-AS-, or Bcl-2-2-mismatch-AS-treated cells were determined (see under "Experimental Procedures"). Bcl-2 levels in control and in Bcl-2-AS-treated cells were 30 ± 6 and 1 ± 0.5 units/mg protein, respectively (n = 4–5 in each case, p < 0.01). BCL-2 levels were not affected significantly in MRP−/− cells or by the addition of anti-CFTR antibodies (not shown). Results obtained by substituting Bcl-2-AS (in Bcl-2-AS- or Bcl-2-AS + anti-CFTR-treated cells) by its 2-base mismatch counterpart were not significantly different from those obtained in controls or in the presence of anti-CFTR alone (not shown). Data are means ± S.D. for 4–5 independent experiments. A two-way ANOVA was used to make comparisons among different treatments and time points. Perfusion time is given in hours. Different superscript letters indicate differences, p < 0.05.

### TABLE II

| Additions          | GSH efflux | GSH  |
|--------------------|------------|------|
|                    | 1 h        | 6 h  |
|                    | nmol/10^6 cells × h | nmol/10^6 cells |
| None               | 1.3 ± 0.1c | 1.3 ± 0.2c |
| Bcl-2-AS           | 1.8 ± 0.3b | 1.7 ± 0.3c |
| VRP                | 2.0 ± 0.3b | 2.1 ± 0.3b |
| Bcl-2-AS + VRP     | 2.4 ± 0.4a | 2.6 ± 0.4a |

![G3PDH](image)  

**FIG. 1.** Effect of monoclonal antibodies anti-CFTR on the GSH release from B16M-F10 cells. B16M-F10 cells were cultured for 24 h. BCL-2 levels in control and in Bcl-2-AS-treated cells were 30 ± 6 and 1 ± 0.5 units/mg protein, respectively (n = 4–5 in each case, p < 0.01). BCL-2 levels were not affected significantly in MRP−/− cells or by the addition of anti-CFTR antibodies (not shown). Results obtained by substituting Bcl-2-AS (in Bcl-2-AS- or Bcl-2-AS + anti-CFTR-treated cells) by its 2-base mismatch counterpart were not significantly different from those obtained in controls or in the presence of anti-CFTR alone (not shown). Data are means ± S.D. for 4–5 different experiments. The significance test (Student’s t test) refers to the comparison of each group versus control values (*, p < 0.05; **, p < 0.01).

GSH at physiological plasma concentrations (see under "Experimental Procedures"). This setup allowed us to use a VRP concentration (1 μM) that corresponds to clinically accepted and nontoxic levels in plasma (45) (dose-response studies showed that 0.5–2 μM VRP activates GSH efflux from perfused cells in a concentration-dependent fashion, data not shown). VRP accumulation within perfused B16M-F10 cells peaked 10–20 min (~70 pmol/10^6 cells) after addition to the perfusate flow and then decreased reaching a lower steady-state concentration at 60 min (~40 pmol/10^6 cells) (Fig. 3). These values of VRP accumulation, which are in agreement with those reported previously in HeLa cells (43), were not changed significantly when control and BCL-2-AS-treated cells were compared (Fig. 3).

As shown in Table II, Bcl-2-AS and VRP independently increased rates of GSH efflux from perfused B16M-F10 cells. In fact, VRP had no effect on GSH efflux in MRP−/− B16M-F10 cells (not shown), and when Bcl-2-AS and VRP were both present their effects were additive and constant along a 12-h perfusion time (GSH efflux was increased by ~100%, Table II). Intracellular GSH contents were significantly decreased (~30%), as compared with controls, in Bcl-2-AS- and VRP-treated B16M-F10 cells after 6 h of perfusion (Table III). However, at 12 h of perfusion time, GSH levels were ~70% higher in Bcl-2-AS- and VRP-treated B16M-F10 cells than in controls (Table II). Thus, it appeared plausible that loss of GSH accelerates their rate of intracellular synthesis.

Antisense Bcl-2 Oligodeoxynucleotides- and/or Verapamil-induced Acceleration of GSH Efflux Associates with γ-Glutamylcysteine Synthetase Overexpression in B16M-F10 Cells—As shown in Table III, either both or Bcl-2-AS or VRP treatment
overexpression.

B16M-F10 cells. [3H]VRP accumulation by control (●) or Bcl-2-AS-treated (○) cells was determined over 720 min of perfusion time (see under “Experimental Procedures”). The concentration of VRP (1 μM in the perfusate flow) was maintained constant during the perfusion. Data points are means ± S.D. of 4–5 independent determinations.

**TABLE III**

GSH synthesis in perfused B16M-F10 cells under conditions of Bcl-2-AS and/or VRP-induced acceleration of GSH release

B16M-F10 cells were cultured and perfused as indicated in the legend to Table II. For GSH synthesis, cells were taken from the perfusion chamber, washed twice, and resuspended in ice-cold Krebs-Henseleit bicarbonate medium, pH 7.4, and incubated (5 mg of dry weight/ml) in 10-ml-Erlenmeyer flasks (final volume 2 ml) for 60 min, at 37°C, in the presence of the amino acid precursors for GSH synthesis (5 mM L-Glu, 2 mM Gly, 1 mM L-Ser, 1 mM N-acetylcysteine). Glucose (5 mM) and bovine serum albumin (2%) were always present. GSH synthesis was calculated from total GSH content at 0, 20, 40, and 60 min of incubation. No significant differences were found when data displayed in the table for 6 h were compared with those obtained at 1 h of perfusion time (not shown). GSH-S activity in controls (see None under the heading Additions) was 15 ± 3 milliunits/10^6 cells (this value did not change significantly along the perfusion time even in the presence of Bcl-2-AS and/or VRP, n = 4–5 in each case, not shown). Results obtained by substituting Bcl-2-AS by its 2-base mismatch counterpart were not significantly different from those obtained under no additions or in the presence of VRP alone (not shown). Data are means ± S.D. for 8–10 independent experiments. Perfusion time was given in hours. A two-way ANOVA was used to make comparisons among groups. Different superscript letters within a column indicate differences, p < 0.01.

| Additions | GSH synthesis | g-GCS activity |
|-----------|---------------|----------------|
|           | 6 h           | 12 h           | 6 h           | 12 h           |
| None      | 1.4 ± 0.3a    | 1.2 ± 0.3a     | 185 ± 24b     | 193 ± 33b      |
| Bcl-2-AS  | 1.2 ± 0.2a    | 2.0 ± 0.2b     | 168 ± 31c     | 255 ± 21b      |
| VRP       | 1.0 ± 0.3b    | 2.5 ± 0.4b     | 176 ± 40c     | 294 ± 47b      |
| Bcl-2-AS + VRP | 1.3 ± 0.4a   | 4.0 ± 0.5a     | 154 ± 28c     | 405 ± 44a      |

significantly increased the rate of GSH synthesis in B16M-F10 cells. Moreover, their effects appeared additive and associated with an increase in g-GCS activity (Table III). Bcl-2-AS and/or VRP, as compared with controls, did not change significantly the GSH synthetase activity (see Table III legend). Moreover, as shown in Table IV, the increase in g-GCS activity was accompanied by a previous increase in both g-GCS-HS and g-GCS-LS expression (maximum values were found at 3 h). Therefore, increased GSH efflux associates with g-GCS overexpression.

Changes in g-GCS activity can result from transcriptional and post-transcriptional regulation affecting the HS and/or the LS (for review see Ref. 46). Intracellular GSH depletion, e.g. that induced by GSH-conjugating agents such as diethyl maleate, increased transcription of both subunits (46) as it occurs in our experimental conditions. Moreover, oxidative stress, which may arise as a consequence of GSH depletion and increased intracellular levels of H_2O_2 and OH·, can also induce increased transcription of both subunits (46). In fact, Bcl-2-AS and VRP-induced GSH depletion (Table II) is accompanied by an increase in H_2O_2 production by the B16M-F10 cells (13 ± 4 in controls and 25 ± 5 nmol of H_2O_2/10^6 cells × h in Bcl-2-AS and VRP-treated cells, n = 5, p < 0.01).

Inhibition of g-Glutamyltranspeptidase Activity Prevents GSH Content Increase in B16M-F10 Cells Treated with Antisense Bcl-2 Oligodeoxynucleotide and Verapamil—In rapidly growing tumors cyste(e)ine, whose concentration in blood is low, may become limiting for GSH synthesis and cell growth (4, 47). Thus, in order to increase the rate of GSH synthesis, malignant cells may require alternative pathways to ensure cyste(e)ine availability.

Keeping the extracellular supply of amino acids constant and physiological during the perfusion (see under “Experimental Procedures”), the intracellular availability of amino acid precursors for GSH synthesis was investigated. The concentrations of free l-glutamate and glycine within the B16M-F10 cells were constant through the perfusion time (e.g. 3.3 ± 0.4 and 2.5 ± 0.3 mM, respectively, in controls; n = 6; enough to ensure maximum rates of GSH synthesis (12)) and were not changed by addition of Bcl-2-AS and/or VRP (not shown). However, free intracellular l-glutamine and l-cyste(e)ine were undetectable, which is not surprising because l-glutamine is a major fuel used by cancer cells (48), and l-cyste(e)ine is rapidly used for protein and GSH synthesis (25). l-Cysteine is predominant outside the cell because l-cysteine rapidly autoxidizes to l-cystine in the extracellular fluids, but once it enters the cell through the Xc- system l-cysteine is reduced to l-cysteine (Ref. 46 and references therein). Thus, we measured l-cysteine uptake by B16M-F10 cells and found that Bcl-2-AS and/or VRP did not significantly affect this rate (e.g. 0.41 ± 0.08 nmol/mg protein in controls, n = 5). Nevertheless, we showed that tumor γ-GT activity and an interstitial flow of GSH increase GSH content in B16M-F10 cells and work as a tumor growth-promoting mechanism (4). γ-GT cleaves extracellular GSH-releasing γ-glutamyl-amino acids and cysteinylglycine, which is further cleaved by membrane-bound dipeptidases into l-cysteine and glycine (12). Free γ-glutamyl amino acids, l-cysteine and glycine, entering the cell serve as GSH precursors (12). Hence, γ-GT expression may provide tumor cells with a growth advantage at physiological concentrations of l-cyste(e)ine (4, 47). Therefore, if the increase in GSH content (Table II) following

| Additions | γ-GCS-HS | γ-GCS-LS |
|-----------|---------|---------|
|           | 6 h     | 12 h    | 6 h     | 12 h    |
| None      | 1.0 ± 0.1a | 1.1 ± 0.2b | 1.2 ± 0.1a | 0.9 ± 0.1a |
| Bcl-2-AS  | 1.9 ± 0.2b | 1.1 ± 0.3b | 1.8 ± 0.3b | 1.0 ± 0.2b |
| VRP       | 2.2 ± 0.3c | 1.4 ± 0.2c | 1.9 ± 0.2c | 1.2 ± 0.1c |
| Bcl-2-AS + VRP | 3.2 ± 0.5c | 1.8 ± 0.3c | 2.7 ± 0.4a | 1.5 ± 0.2b |

FIG. 3. Time course of [3H]verapamil uptake by perfused B16M-F10 cells. [3H]VRP accumulation by control (●) or Bcl-2-AS-treated (○) cells was determined over 720 min of perfusion time (see under “Experimental Procedures”). The concentration of VRP (1 μM in the perfusate flow) was maintained constant during the perfusion. Data points are means ± S.D. of 4–5 independent determinations.

TABLE IV

Acceleration of GSH release associates with γ-GCS overexpression in perfused B16M-F10 cells

B16M-F10 cells were cultured and perfused as described in the legend to Table II. γ-GCS-HS and γ-GCS-LS expression was determined after 3 and 6 h of perfusion. Results obtained by substituting Bcl-2-AS by its 2-base mismatch counterpart were not significantly different from those obtained under no additions or in the presence of VRP alone (not shown). The figures, expressing fold induction, show mean values ± S.D. from 5 to 6 different experiments. Perfusion time is given in hours. A two-way ANOVA was used to make comparisons among groups. Different superscript letters within a column indicate differences, p < 0.05.
the effect of Bcl-2-AS and VRP on GSH efflux depends on L-cyst(e)ine availability, and if this is provided in part by the γ-GT, then inhibition of this activity could limit GSH synthesis. We tested this possibility by adding to the perfusion system ACV, an irreversible γ-GT inhibitor (49). ACV was present in the perfusate flow for only 30 min (Fig. 4). ACV accumulation within the B16M-F10 cells peaked 10–20 min after addition, and then its concentration decreased to a steady-state low level (∼4 pmol/10^6 cells) (Fig. 4; these values were not changed when control and Bcl-2-AS- or VRP-treated cells were compared, data not shown). ACV decreased γ-GT activity to nondetectable levels and decreased GSH synthesis but did not affect the rate of GSH efflux (Table V) or the rate of L-cysteine uptake (not shown, see above for control values). However, the rate of GSH synthesis in B16M-F10 cells treated with Bcl-2-AS, VRP, and ACV was found similar to controls (as under “no additions” in Table V) when the concentration of L-cysteine in the perfusion buffer was increased 2-fold (up to 16 μM). Hence, intracellular L-cysteine availability indeed appears modulated by its γ-GT-dependent generation from extracellular GSH. Therefore, we conclude that Bcl-2-AS- and VRP-induced acceleration of GSH efflux combined with inhibition of γ-GT promote GSH depletion in B16M-F10 cells.

FIG. 4. Time course of [3H]acacivicin uptake by perfused B16M-F10 cells. [3H]ACV accumulation by control (●) or Bcl-2-AS-treated (○) cells was determined over 720 min of perfusion time (see under “Experimental Procedures”). [3H]ACV (1 μM) was added to the perfusate flow at 30 min of perfusion and was present for 30 min. Data points are means ± S.D. of 4–5 independent determinations.

Effect of ACV-induced inhibition of γ-GT on GSH content in perfused B16M-F10 cells treated with Bcl-2-AS and VRP

B16M-F10 cells were cultured and perfused as indicated in the legend to Table II. VRP addition and concentration in the perfusate flow are shown in Table II and Fig. 3. ACV concentration in the perfusate flow was 1 μM (see Fig. 4 and the text for details regarding its cellular pharmacokinetics). ACV was added to the perfusate flow as indicated in the legend to Fig. 4. GSH content and γ-GT activity were measured at 12 h of perfusion time. GSH synthesis and efflux were measured, starting at 12 h of perfusion, as indicated in the legend to Table III but using a complete mixture of amino acids as precursors. This mixture contained plasma concentrations (aortic blood from non-tumor-bearing C57BL/6J mice) × 10 of GSH and free L-amino acids (see under “Perfusion of B16M-F10 Cells” under “Experimental Procedures”). Results obtained by substituting Bcl-2-AS by its 2-base mismatch counterpart were not significantly different from those obtained in the presence of VRP or VRP + ACV (not shown). Data are means ± S.D. for 5–6 different experiments. A one-way ANOVA was performed for comparison among groups. Different superscript letters within a column indicate differences, p < 0.01.

| Additions        | γ-GT (milliunits/10^6 cells) | GSH synthesis (nmol/10^6 cells × h) | GSH efflux (nmol/10^6 cells) | GSH   |
|------------------|-----------------------------|------------------------------------|-----------------------------|--------|
| None             | 33 ± 6^a                    | 1.1 ± 0.2^a                         | 1.4 ± 0.2^a                 | 26 ± 4^b |
| Bcl-2-AS + VRP   | 39 ± 8^a                    | 4.2 ± 0.4^a                         | 2.9 ± 0.4^a                 | 40 ± 6^a |
| ACV              | 0 ± 0                       | 0.6 ± 0.2d                          | 1.6 ± 0.3^b                 | 19 ± 2c  |
| Bcl-2-AS + VRP + ACV | 0 ± 0                       | 1.9 ± 0.3b                          | 3.1 ± 0.5^a                 | 12 ± 3^d  |
B16M-F10 cells were previously cultured for 3 days in the presence or in the absence of 50 μM Bcl-2-AS as indicated in the legend to Table III. The cells were then harvested and cultured again in the absence of oligodeoxynucleotides. Twenty-four hour cultured HSE cells (~2.5 × 10^5 cells/well) were co-cultured with B16M-F10 cells (~5.0 × 10^5 cells/well; precultured for 24 h). Twenty minutes after B16M-F10 addition to the HSE, the plates were washed as described under “Experimental Procedures.” The ratio of tumor cells adhering to the HSE was ~1:1. TNF-α (100 units/ml) and IFN-γ (50 units/ml), used as a potent activators of NO and H2O2 generation by the HSE (3), were added to the co-cultures when all tumor cells present were attached to the HSE. In endothelium-induced B16M-F10 cytotoxicity assays, tumor cytotoxicity (expressed as the percentage of tumor cells that lost viability within the 4–6-h period of incubation, see “Experimental Procedures”) was determined after 6 h of incubation. During the 6-h period of incubation, the percentage of HSE cell viability was 98–99% in all cases. When adding TNF-α (100 units/ml) and IFN-γ (50 units/ml) to cultured B16M-F10 cells, no cytostatic or cytotoxic effects were observed within the next 6 h. When indicated B16M-F10 cells were incubated for 4 h with GSH ester (1 mM), BSO (0.2 mM), VRP (1 μM), or ACV (1 μM) before co-culture with endothelial cells. Pretreatment of B16M-F10 cells with Bcl-2-AS, VRP, or ACV individually did not affect significantly control values of tumor cell adhesion. Pretreatment of B16M-F10 cells with Bcl-2-AS alone increased tumor cell cytotoxicity to ~36%, either in the presence or in the absence of GSH ester. Compared with controls, pretreatment of B16M-F10 cells with VRP or ACV did not affect significantly the percentage of tumor cytotoxicity. BCL-2 levels in Bcl-2-AS-treated cells were always <4 units/mg protein. Data are means ± S.D. for 5–6 independent experiments. A two-way ANOVA was used to make comparisons among different treatments. Different superscript letters indicate differences, p < 0.01.

### Table VI

| Pretreatment of B16M-F10 cells | B16M-F10 GSH content (nmol/10^5 cells) before co-culture with HSE cells | Adhesion (%) | Cytotoxicity (%) |
|-------------------------------|-------------------------------------------------|--------------|-----------------|
|                               | −GSH ester                                      | +GSH ester   | −GSH ester       | +GSH ester   |
| Control                       | 35 ± 4a                                         | 48 ± 5a      | 55 ± 7c          | 51 ± 4c      |
| BSO                           | 14 ± 5c                                         | 45 ± 4c      | 50 ± 5c          | 53 ± 6c      |
| Bcl-2-AS + VRP + ACV          | 12 ± 5c                                         | 44 ± 4c      | 52 ± 7c          | 54 ± 6c      |

**DISCUSSION**

Analysis of a Bcl-2 family of genes revealed that B16M-F10 cells (high metastatic potential), as compared with B16M-F1 cells (low metastatic potential), preferentially overexpressed Bcl-2 (5). BCL-2 overexpression, without changing the rate of GSH synthesis, induces a decrease in GSH efflux and, consequently, an increase of GSH content within B16M-F10 cells (5). Most B16M cells with a high GSH content survive the NO- and H2O2-mediated tumoricidal activity of endothelial cells (50). However, survival of B16M-F10 during interaction with the vascular endothelium can be challenged by inhibiting their BCL-2 and GSH synthesis in vitro (5). Bcl-2 antisense therapy using G3139, for example, an 18-base phosphorothioate oligonucleotide complementary to the first six codons of the Bcl-2 mRNA, selectively and specifically inhibits BCL-2 expression and promotes apoptosis in different human and murine cancer cell lines (51). Systemic administration of G3139 to Shionogi tumor-bearing mice led to a rapid decrease of tumor size (higher when chemotherapy was simultaneously administered), whereas the oligonucleotide did not affect BCL-2 expression in normal organs (20, 52). G3139-induced tumor regression without dose-limiting toxicity was also observed in other tumors, melanoma, lymphoma, or gastric cancers for example (51). Furthermore, synergism of the G3139 and anticancer drugs was also shown in different tumors (53–55). However, on the other hand, an effective strategy to deplete GSH in metastatic cells has remained elusive.
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B16M-F10 cells surviving the combined nitrosative/oxidative attack induced by the HSE showed a decrease in GSH content (50). However, endothelial NO-mediated partial inactivation of γ-GCS activity is followed by overexpression of γ-GCS-heavy and -light subunits, which leads to a rapid recovery of GSH levels within invasive cells (50). Therefore, as suggested by previous achievements in nonmetastatic models (6), a methodology capable of maintaining low GSH levels in metastatic cells could represent a critical advance in cancer therapy.

Here we present evidence showing that GSH is released from highly metastatic B16M-F10 cells through MRPI and CFTR (Table I and Fig. 1). By using a perfusion system that mimics in vivo conditions, we show that GSH efflux from B16M-F10 cells can be accelerated be using Bcl-2-AS (which prevents BCL-2-induced inhibition of GSH release through CFTR) and VRP (which activates GSH release through MRPI (42)) (Tables I and II). However, Bcl-2-AS and/or VRP treatment associated with overexpression of γ-GCS (Table IV) increased rates of GSH synthesis (Table III) and higher GSH content (Table II) within the B16M-F10 cells.

Recently, we showed that tumor γ-GT activity, by providing an extra supply of L-cysteine from extracellular GSH, supports GSH synthesis in B16M-F10 cells and promotes their metastatic growth (4). Hence, we tested whether inhibition of this activity could prevent the increase of GSH content within BCL-2-AS- and VRP-treated B16M-F10 cells. Indeed, as shown in Table III, ACV limited GSH synthesis and allowed GSH efflux to deplete tumor cell GSH. This strategy, which combines inhibition of BCL-2 and GSH depletion, sensitized perifused B16M-F10 cells to the cytotoxic effects of the vascular endothelium (Tables VI and VII).

Can possible clinical applications be derived from our study? The proto-oncogene Bcl-2 and its anti-apoptotic homologs are mitochondrial membrane permeabilization inhibitors (56) and participate in the development of chemoresistance (57), whereas expression of pro-death genes, e.g. Bax or Bak, is often reduced in cancer cells (58). In agreement with this idea, Takaoka et al. (59) observed that Bcl-2 overexpression in B16M cells enhanced pulmonary metastasis. In fact, a major form of multidrug resistance in human tumors is caused by overexpression of the MRPI gene (7). In vivo Bcl-2-AS therapy, as explained above, is feasible. In addition, VRP (at doses that promote a similar plasma concentration than that used in our experiments) has already been used in cancer patients with myeloma or acute lymphocytic leukemia, for example, where it increased accumulation of daunorubicin or vincristine within the tumor cells (60). ACV, the L-glutamine analog anti-metabolite, has followed phase I and II clinical trials in different tumors (61). Although its use is limited by severe central nervous system toxicity, a maximum tolerated dose of 50 mg of acivicin/m²/day has been proposed in combination with the amino acid solution aminosyn (which decreases drug uptake in the central nervous system) (61). In our studies, ACV concentration in the perfusate flow was 1 μM (present only during 30 min) (Fig. 4 and Table III). By taking into account the circulating blood volume and the in vivo pharmacokinetics in humans (61), this means that ACV doses required to block tumor γ-GT activity will remain within nontoxic levels. This is important because an increased expression of γ-GT has been found in melanoma as well as in other cancers (including human tumors of the liver, lung, breast, and ovary) (62).

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