ANTIOXIDANTS & REDOX SIGNALING
Volume 15, Number 9, 2011

DOI: 10.1089/ars.2010.3830

ORIGINAL RESEARCH COMMUNICATION

The Cystine/Cysteine Cycle and GSH Are Independent and Crucial Antioxidant Systems in Malignant Melanoma Cells and Represent Druggable Targets

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Abstract

Aims: Cancer chemoresistance is often due to upregulation of antioxidant systems. Therapeutic targeting of these systems is however hampered by their redundancy. Here, we have performed a functional dissection of the antioxidant systems in different melanoma cases aimed at the identification of the most effective redox active drug. ***Results:*** We have identified two crucial antioxidant mechanisms: glutathione (GSH), the major intracellular redox buffer, and the cystine/cysteine cycle, which switches the extracellular redox state from an oxidized to a reduced state. The two mechanisms are independent in melanoma cells and may be substitutes for each other, but targeting both of them is lethal. Exposure to the pro-oxidant compound As_2O_3 induces an antioxidant response. However, while in these cells the intracellular redox balance remains almost unaffected, a reduced environment is generated extracellularly. GSH depletion by buthioninesulfoximine (BSO), or cystine/cysteine cycle inhibition by (*S*)-4-carboxyphenylglycine (sCPG), enhanced the sensitivity to As_2O_3. Remarkably, sCPG also prevented the remodeling of the microenvironment redox state. ***Innovation:*** We propose that the definition of the prevalent antioxidant system(s) in tumors is crucial for the design of tailored therapies involving redox-directed drugs in association with pro-oxidant drugs. ***Conclusion:*** In melanoma cells, BSO is the best enhancer of As_2O_3 sensitivity. However, since the strong remodeling of the microenvironmental redox state caused by As_2O_3 may promote tumor progression, the concomitant use of cystine/cysteine cycle blockers is recommended. *Antioxid. Redox Signal.* 15, 2439–2453.

Introduction

Reactive oxygen species (ROS) are physiologically produced by aerobic cells at a low level as a result of electron transfer reactions (13), and their production increases under conditions of stress, either internal or external (51). ROS mediate crucial signaling pathways and are essential for cell survival. However, an excess of ROS generates damage and eventually cell death. To prevent these outcomes, the increase of ROS induces an adaptive response, consisting of a compensatory upregulation of antioxidant systems aimed to restore the redox homeostasis (6).

Several highly redundant enzymatic systems are involved in the maintenance of the intracellular redox balance. Among these, a main role is played by the glutathione (GSH) (34) and thioredoxin (28) systems, In addition, the cystine/cysteine redox cycle has been recently proposed as a major antioxidant mechanism as well (3). The latter has been found highly active in primary inflammatory cells (2, 48, 49, 52, 57) and in tumor cells of various origins (30), including lymphoma (2), lung (7), pancreas (29), and prostate (12). The cystine/cysteine redox cycle is composed of the x_c^- cystine/glutamate antiporter (42) and oxidoreductases such as thioredoxin (32). The x_c^- antiporter mediates uptake of cystine, the oxidized form of the amino acid cysteine that prevails extracellularly. Thioredoxin and possibly other reductases mediate the conversion of cystine to reduced cysteine that is then externalized, resulting in a switch of the extracellular redox from the oxidizing physiologic state to a reduced state. Therefore, unlike most antioxidant systems that primarily modify the intracellular redox, the upregulation of cystine/cysteine redox cycle acts also by affecting the extracellular redox. Whether this cycle is functionally linked to GSH or the two systems work autonomously is still debated (1, 3, 32, 57).

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Innovation

Redundant antioxidant mechanisms overexpressed by neoplastic cells contribute to their aggressiveness and chemoresistance. The definition of the prevailing antioxidant system(s) in single tumors is therefore mandatory for the choice of the most effective redox active drug. The findings of this study have identified in melanoma cells from different patients two crucial and independent antioxidant systems, glutathione and the cystine/cysteine cycle, and demonstrated that targeting both of them is lethal for melanoma cells. Moreover, it was found that cells with low expression of the cystine/cysteine cycle component xCT are more sensitive to therapeutic doses of As_2O_3. However, a fraction of cells survive to this treatment and trigger an adaptive response with upregulation of the cystine/cysteine cycle resulting in a strong reduction of the tumor microenvironment. A reduced extracellular redox state may promote tumor progression through various mechanisms, including increase of damage associated molecular pattern molecules activity. Hence, it is proposed that the association with redox-active drugs targeting the cystine/cysteine cycle not only increases pro-oxidant drug toxicity but also avoids detrimental effects accomplished by surviving cells.

In the past decade, *in vitro* and *in vivo* studies have indicated that the cystine/cysteine redox cycle plays an important role in various aspects of cancer. xCT, the functional subunit of the x_c^- antiporter, is overexpressed in tumor cell lines of various origin and in primary human cancers (29), and many tumor cells, including pancreatic and glioma neoplastic cells, depend on x_c^--mediated cystine uptake for growth (11, 30). The role of x_c^- in cancer growth and progression has been recently supported by the observation that loss of xCT from the cell surface suppresses tumor growth in a transgenic mouse model of gastric cancer (20). Interestingly, xCT is also involved in metastasis formation (10). In the light of these observations, the x_c^- cystine/glutamate antiporter represents an appealing new target for therapy of cancer (30). Accordingly, sulfasalazine, a drug that blocks the uptake of cystine through x_c^-, has displayed promising antineoplastic effects both *in vitro* and *in vivo* (11, 12, 31).

Oxidative stress has long been implicated in cancer development and progression (19), suggesting that antioxidants may protect from cancer (4). However, the efficacy of a preventive or therapeutic approach with antioxidants has been recently questioned (25). On the other hand, pro-oxidant therapies, including ionizing radiations and chemotherapeutic agents, are widely used in clinics based on the rationale that a further oxidative hit summed to the constitutive oxidative stress in tumor cells should cause the collapse of the antioxidant systems, leading to cell death (26, 50). However, this approach also provided unsatisfactory results (54). One of the reasons is that many primary tumors overexpress reducing enzymes at very high levels, resulting in resistance to pharmacologically sustainable drug doses (39, 46, 54, 58). Alternatively, even if most neoplastic cells die, a cell fraction displaying a strong upregulation of antioxidant systems, preexistent or induced by the pro-oxidant drug, may survive the treatment (22, 44). The selection of resistant cells with a highly reduced redox phenotype represents an additional constraint to the use of pro-oxidant drugs since overexpression of antioxidant systems in tumors correlates with poor prognosis (46). Accordingly, we have recently shown that the activity of the cystine/cysteine redox cycle is associated with the growth rates of lung cancer cells *in vitro* and *in vivo*, and that the more aggressive behavior of such cells correlates with the more reducing phenotype (7). Moreover, the same dose of the pro-oxidant agent As_2O_3 was able to kill tumor cell lines featured by a more reduced phenotype but induces upregulation of antioxidant systems and increased proliferation rate on cell line displaying a less reduced redox state (7).

Human malignant melanoma is the most serious skin cancer, responds poorly to conventional chemo- and radiotherapy, and leads rapidly to death in the advanced stages (35). The potential mechanisms for poor response include the high degree of resistance of melanoma cells to oxidative stress (15, 55). In recent years, many efforts have been undertaken to develop more efficient therapeutic protocols. Inhibition of heme oxygenase-1, which confers cytoprotection against oxidative injury, has been shown to increase the responsiveness of melanoma cells to amino-levulinic acid-based photodynamic therapy (14). Promising results have also been obtained with the association of As_2O_3 with inhibitors of survival pathways (21) or with ascorbic acid (15). Interestingly, the latter was successful in melanoma cell clones with low, but not with high, expression of γ-glutamyltransferase, supporting the concept that high level of expression of antioxidant enzymes and redundancy of the antioxidant systems represent a major limit to the use of redox-directed drugs in association with anti-tumor therapies (56).

To find the best molecular target among overexpressed antioxidant systems in melanoma, we have studied the redox phenotypes of cell clones derived from three metastatic human melanoma patients and the redox remodeling induced by the pro-oxidant drug As_2O_3. Our data show that the prevalent antioxidant systems, as well as the adaptive response induced by As_2O_3, overlap largely, although not completely, in the single melanoma cases and indicate that the definition of the redox phenotype is crucial for the choice of the best association between chemotherapeutic and redox active drugs.

Results

Redox phenotype of human melanoma cells

Cell lines from three human metastatic melanoma cases were generated *in vitro* (5) and the mechanisms of redox adaptation operating in these cells were investigated. The three cell lines (Me-CA, Me-PA, and Me-MOR) produced ROS at different extents, with Me-CA cells displaying more than twice ROS than Me-PA cells, while Me-MOR cells exhibited intermediate ROS levels (Fig. 1A). Regarding the antioxidant systems, we first focused on GSH, the major intracellular antioxidant and ROS-scavenging system (34), and on the cystine/cysteine redox cycle, as a cancer-related mechanism modulating both intra- and extracellular redox (3, 32). As shown in Figure 1B, the levels of intracellular reduced GSH, evaluated by MCB staining, were only slightly different in the three melanoma cell lines. lnterestingly, the three cell lines displayed significantly different expression and activity of the cystine/cysteine redox cycle. The highest levels of xCT expression (Fig. 1C) and of spontaneous extracellular release of

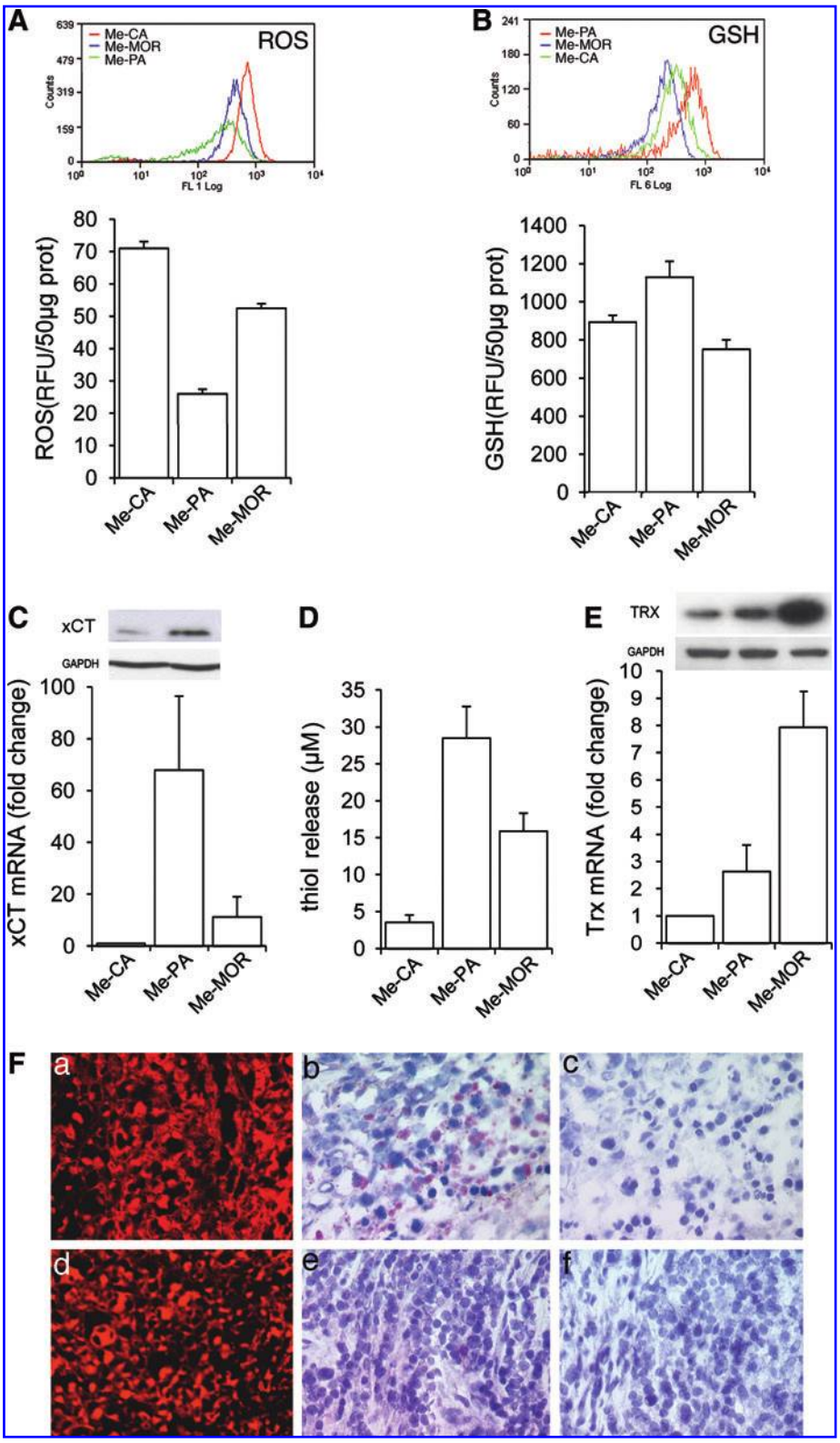

FIG. 1. Redox phenotype of melanoma cells. Intracellular ROS **(A)** and reduced GSH **(B)** levels, expression of xCT **(C)**, cysteine release **(D)**, and expression of thioredoxin **(E)** were compared in Me-CA, Me-PA, and Me-MOR cells as indicated. **(A and B)**: ROS **(A)** and reduced GSH **(B)** levels were quantified by flow cytometry of intact cells loaded with H_2DCFDA and MCB respectively (*upper panels* show representative results) or by fluorimetry of cell lysates (*lower panels*; data are expressed as relative fluorescence units (RFU), mean ± SD ($n = 3$)). Representative results from one of three independent experiments are shown. **(C)** Real-time PCR analysis of the expression of mRNA for xCT. Data are expressed as fold changes with respect to Me-CA; mean ± SD of data from several experiments are shown; *inset:* Western blot analysis of xCT protein content in cell lysates from the different cell lines. **(D)** Extracellular thiols were quantified by DTNB assay in 24 h supernatants. Data are expressed as *µM*. Representative results from one of three independent experiments are shown as the mean ± SD of triplicates. **(E)** Real-time PCR analysis of the expression of mRNA for thioredoxin (Trx). Data are presented as fold changes with respect to Me-CA; *inset:* Western blot analysis of thioredoxin protein (TRX) content in cell lysates from the different cell lines. **(F)** Mercury Orange (a, d), xCT (b, e) staining of two selected melanoma surgical sample; c and f: control Ab staining. Magnification 400X. (To see this illustration in color, the reader is referred to the web version of this article at www.liebertonline.com/ars).

free thiols (Fig. 1D) were detected in Me-PA cells and the lowest in Me-CA cells. Thioredoxin was expressed by all the cell lines, being more abundant in Me-MOR (Fig. 1E). The thioredoxin real-time PCR data were substantiated by the Western blot analysis (inset). In the case of xCT, a weak specific band was detected in Me-CA and Me-PA cell lysates, of intensity consistent with the mRNA data (Fig. 1C, inset). In contrast, an xCT band displaying the same migration rate was absent from Me-MOR cell lysates, possibly due to the presence of abnormal post-translational modifications or of a variant molecular form of xCT (not shown). The expression of xCT was then investigated on human melanoma surgical

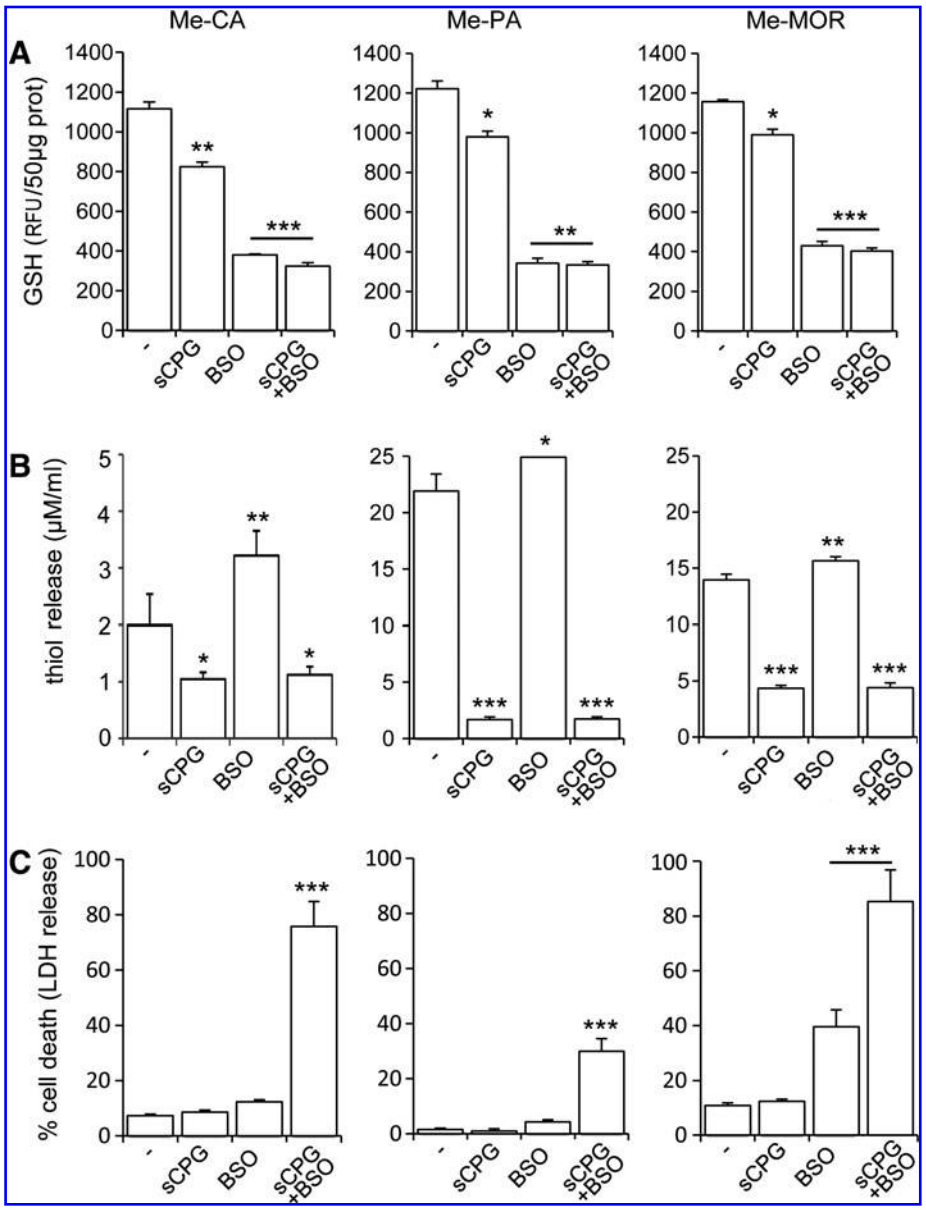

FIG. 2. GSH and the cystine/cysteine cycle have a main role in maintaining redox homeostasis in melanoma cells. GSH content **(A)**, thiol secretion **(B)**, and LDH release **(C)** were evaluated in the three cell lines cultured in the absence (-) or presence of 100 μM BSO (BSO) or 100 μM sCPG (sCPG) or of a combination of the two compounds (sCPG + BSO). **(A)** GSH content after 24 h was quantified by the MCB fluorometric method. Similar results were obtained after 48 h treatment (not shown). Data are expressed as in legend to Figure 1. **(B)** Extracellular thiols after 24 h of treatment were quantified by DTNB assay. Similar results were obtained after 48 h treatment (not shown). Data are expressed as in legend to Figure 1. **(C)** LDH release as a marker of cell death was measured in cell supernatants after 72 h of treatment. Representative results from one of three independent experiments are shown. Significant differences between each treated group with respect to untreated (-) are indicated. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

specimens by immunohistochemistry. Analyses of four melanoma cases showed that the expression of xCT was strong in one, low but well detectable in another, and faint in two of them (Fig. 1F shows the most [b] the less positive [e] cases). Mercury Orange was strongly positive in all cases (Fig. 1F, a and d), confirming the high abundance of non-protein thiols (GSH or cysteine). These data demonstrate that the redox phenotype observed in cultured melanoma cells is not restricted to *in vitro* derived cell lines but is observed also in primary melanoma.

The cystine/cysteine cycle and GSH are independent antioxidant systems and may substitute for each other. Suppression of both is lethal for melanoma cells

To assess the reciprocal interaction between GSH and the cystine/cysteine cycle, we studied the effect of BSO, a specific inhibitor of γ-glutamyl synthase (17), and of sCPG, that blocks cystine uptake through xCT (30), on GSH content, thiol release and cell viability. As shown in Figure 2, in all the three cell lines sCPG decreased only slightly the GSH content (< 25%, panel A), while profoundly inhibited the release of free thiols (> 60%, panel B). In contrast, BSO strongly affected GSH (panel A), that decreased up to 60%, but did not inhibit, and rather induced, thiol release (panel B). The data indicate that less than 25% of cystine taken up through the xCT transporter is used for GSH biosynthesis.

Neither sCPG nor BSO affected significantly cell viability of Me-CA and Me-PA evaluated at 72 h, while BSO, but not sCPG, was slightly toxic for Me-MOR (Fig. 2C). In contrast, simultaneous treatment with both BSO and sCPG dramatically affected the vitality of the three melanoma cell lines, although at a lower extent in Me-PA (Fig. 2C), indicating that GSH and the cystine/cysteine cycle are crucial antioxidant systems in multiple melanoma, that substitute each other but cannot be replaced with other antioxidant systems.

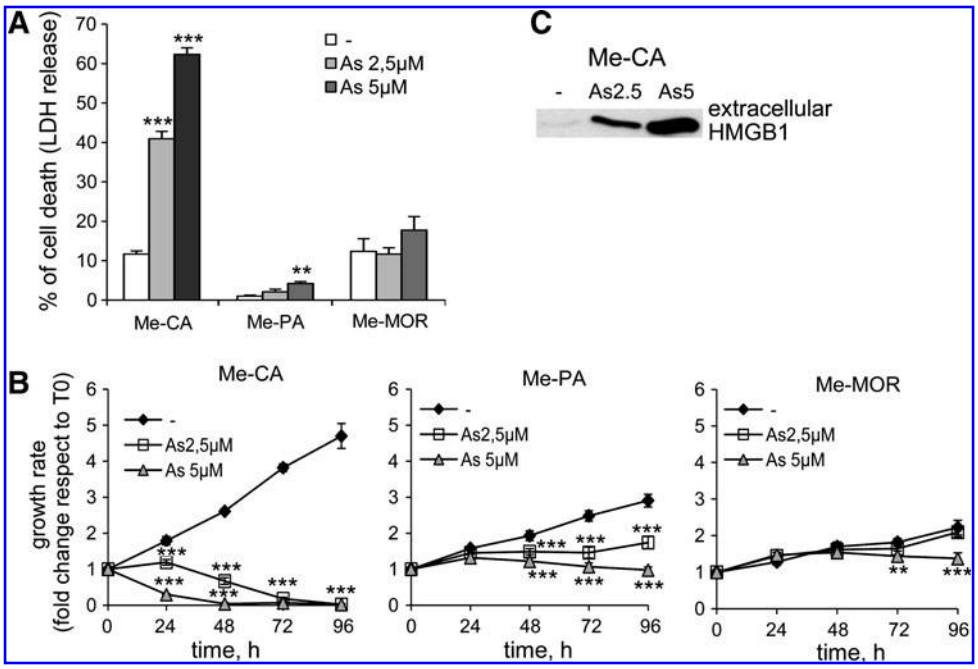

FIG. 3. The cytotoxic effect of As_2O_3 varies in the three melanoma cell lines. (A) LDH release as a marker of cell death was measured in cell supernatants after 48 h of culture in the absence (-) or presence of 2.5 or 5 μM As_2O_3 (As) as indicated. Representative results from one of three independent experiments are shown. For each cell line, significant differences between As_2O_3-treated groups with respect to untreated (-) are indicated; $^{**}p < 0.01$; $^{***}p < 0.001$. **(B)** The growth rate of the three cell lines cultured in the absence (-) or presence of 2.5 or 5 μM As_2O_3 (As) for 24, 48, 72, and 96 h was determined by Crystal Violet staining. Representative results from one of three independent experiments are shown; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. **(C)** Western blot analysis of HMGB1 released in Me-CA cell supernatant after 48 h of culture without (-, *lane 1*) or with 2.5 μM As_2O_3 (*lane 2*), or 5 μM As_2O_3 (*lane 3*). Representative results from one of three independent experiments are shown.

Resistance to As_2O_3 treatment correlates with high activity of the cystine/cysteine cycle

To investigate the therapeutic effects of pro-oxidant drugs on melanoma cells, the three cell lines were exposed to low micromolar doses of As_2O_3. The treatment was well tolerated by Me-PA and Me-MOR that displayed only a slight toxicity at the As_2O_3 doses used (Figs. 3A and 3B). At variance, Me-Ca cells were highly sensitive to As_2O_3, displaying a mortality of 40% to > 60% after 48 h of exposure to 2,5 and 5 μM of As_2O_3, respectively (Fig. 3A). Cell death most likely occurred by necrosis, since less than 5% of As_2O_3-treated cells underwent apoptosis (as demonstrated by annexin V staining, not shown). As_2O_3 treatment also resulted in a dose-dependent release of the intracellular protein HMGB1 in supernatants (Fig. 3C). Since HMGB1 is retained by apoptotic cells and released upon cell lysis (42), the data supports that Me-CA cell death in response to As_2O_3 mostly occurred by necrosis.

The As_2O_3-sensitive Me-CA cells display the lowest activity of the cystine/cysteine cycle (see Figs. 1C and 1D), suggesting that this antioxidant system may be involved in the resistance to As_2O_3. To investigate this point, the redox modulation that follows exposure to As_2O_3 was studied. In agreement with recent results on different tumor cells (9), ROS production was not induced, but rather slightly decreased, by As_2O_3 treatment in all cell lines (Figs. 4A and 4C, upper panel). GSH levels, both reduced (Figs. 4B and 4C, lower panel) and total (Supplementary Fig. S1; Supplementary Data are available online at www.liebertonline.com/ars) were only little affected, whereas all cell lines displayed upregulation of the cystine/cysteine cycle, with increased expression of xCT (Fig. 4D), and, to a lesser extent, of thioredoxin (Fig. 4E), and a strong enhancement of extracellular thiol release (Fig. 4F). As shown in Figure 4G, the major thiol form released by the three cell lines, untreated and after As_2O_3 treatment, is cysteine, in agreement with previous reports on different cell systems (2, 3, 32, 49). Only in the case of Me-PA cells, a little fraction of the released thiols (14%) consists of GSH, and increases slightly following As_2O_3 treatment (17%). Based on these results, from here on we will refer to extracellular thiols as extracellular cysteine.

The extracellular redox state is more modified by As_2O_3 treatment than the intracellular one

The unchanged levels of GSH might suggest that the GSH system is not involved in the response to As_2O_3. Alternatively, the detection of unmodified GSH levels after exposure to As_2O_3 may be due to increased GSH synthesis concomitant to increased GSH oxidation (*i.e.*, decreased GSH/GSSG ratio) or to increased GSH reduction in the absence of GSH synthesis. As shown in Figure 5A, GSH synthase, the enzyme required for GSH biosynthesis (34), was expressed at different extents in the three cell lines and slightly modulated by As_2O_3. GSH reductase (34), responsible for the conversion of oxidized to reduced GSH, was also expressed at different extents, but with an inverse proportion with respect to GSH synthase and also little modulated by As_2O_3 (Fig. 5B). These data suggest that, in melanoma cells, GSH participates in buffering the oxidative hit provided by As_2O_3 but is maintained at the levels displayed in untreated cells, thanks to a balanced action of GSH synthase and reductase (the latter more efficient in Me-PA and Me-MOR).

Other antioxidant systems that are crucial for the maintenance of the redox balance under conditions of oxidative stress, (*e.g.*, glutaredoxins and peroxiredoxins (23)) were not significantly modulated by As_2O_3 (data not shown). Superoxide dismutase-1 expression was only slightly

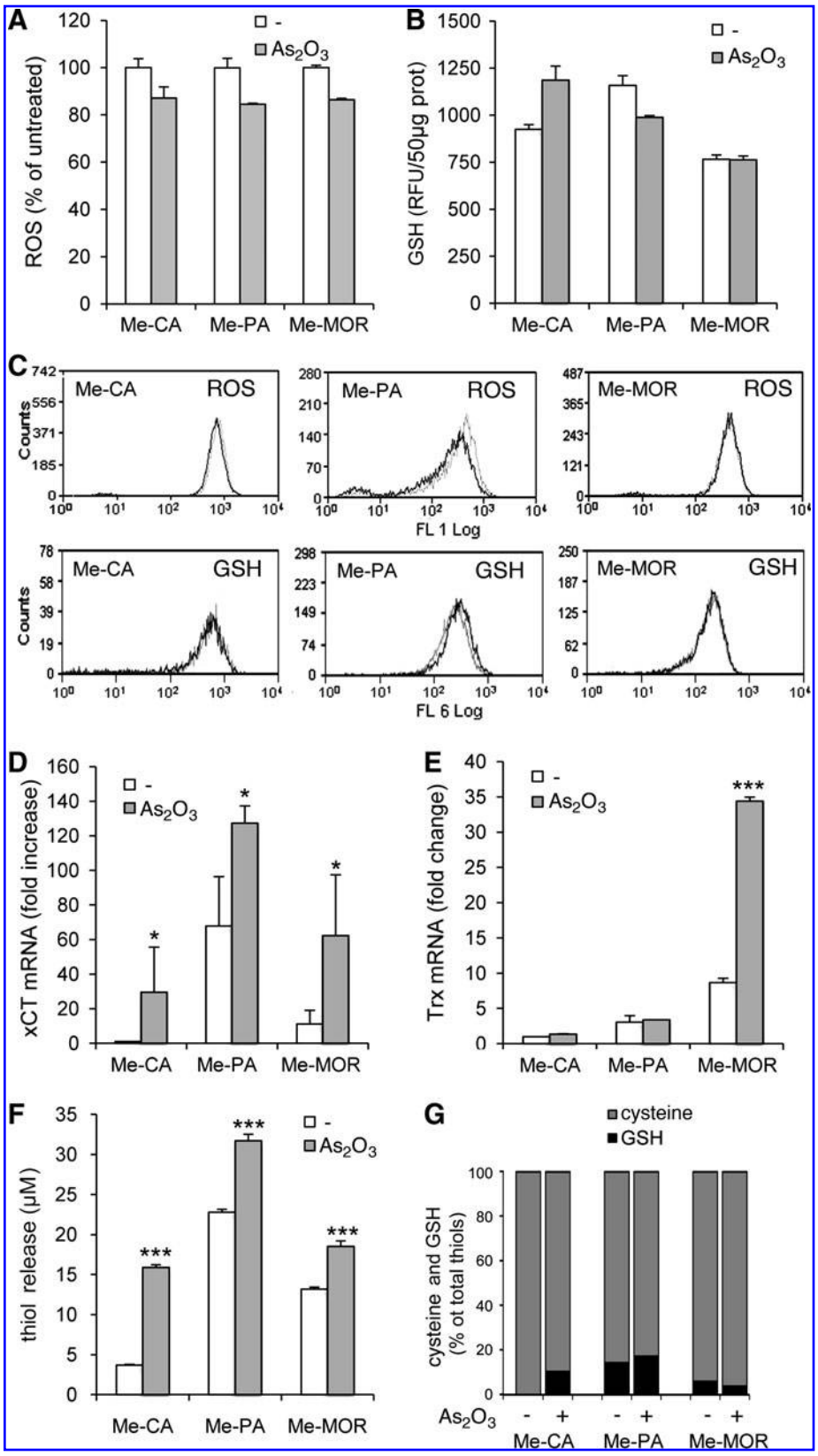

FIG. 4. As_2O_3 induces upregulation of the cysteine/cysteine cycle but does not affect ROS and GSH levels. Intracellular ROS **(A, C** *upper panel***)** and GSH **(B, C** *lower panel***)** levels, expression of xCT **(D)** and thioredoxin (Trx) **(E)**, and thiol release **(F)** were measured as in Figure 1 after treatment in the absence or presence of 2.5 μM As_2O_3 (in the case of Me-CA cells) or 5 μM As_2O_3 (in the case of Me-PA and Me-MOR) as indicated. **(A)** ROS levels were evaluated in cell lysates after 6 h of As_2O_3 treatment. Data are expressed as percent of ROS with respect to untreated cell (-). Representative results from one of three independent experiments are shown. Similar results were obtained after 1, 3, and 24 h treatment (not shown). **(B)** GSH content was evaluated in cell lysates after 24 h As_2O_3 treatment. Data are expressed as in legend of Figure 1B. Representative results from one of three independent experiments are shown. **(C)** ROS (*upper panel*) and GSH (*lower panel*) evaluated by flow cytometry in intact cells, untreated (*black line*) or treated (*gray line*) with As_2O_3 as in Figures. 4A and 4B. A representative experiment is shown. **(D)** Real-time PCR analysis of the expression of mRNA for xCT was performed after 24 h of treatment. Data are expressed as fold changes with respect to untreated Me-CA. For each cell line, significant differences between As_2O_3 treated with respect to untreated (-) are indicated; $^*p < 0.05$. **(E)** Real-time PCR analysis of the expression of mRNA for TRX was performed after 24 h of treatment. Data are expressed as fold changes with respect to untreated Me-CA. For each cell line, significant differences between As_2O_3 treated with respect to untreated (-) are indicated; $^{***}p < 0.001$. **(F)** Extracellular thiol release was evaluated after 24 h of As_2O_3 treatment. For each cell line, significant differences between As_2O_3 treated with respect to untreated (-) are indicated; $^{***}p < 0.001$. **(G)** Percent of cysteine and GSH in supernatants from untreated or 24 h As_2O_3-treated melanoma cells. Data were obtained by HPLC analyses of supernatants and are expressed as percent of cysteine or GSH with respect to total thiols.

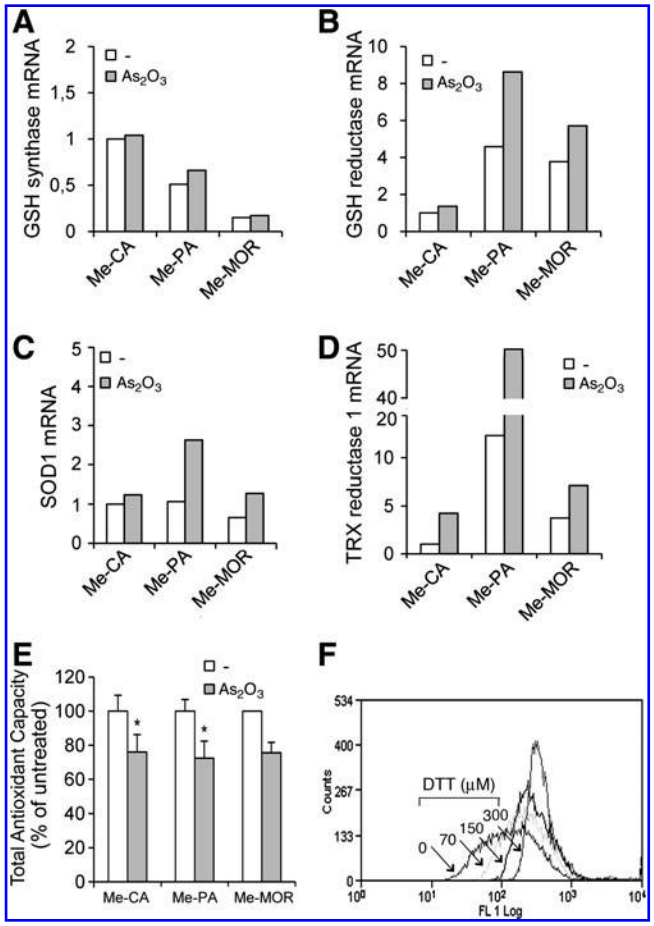

FIG. 5. As_2O_3-induced redox remodeling does not increase the total intracellular antioxidant capacity. (A–D) Real time PCR analysis of the expression of mRNA for GSH synthase **(A)**, GSH reductase **(B)**, SOD1 **(C)**, and thioredoxin (Trx) reductase-1 **(D)** after 4 h of incubation in medium alone (-, *white columns*) or with As_2O_3 (at 2.5 μM for Me-CA and 5 μM for Me-PA and Me-MOR). Data are expressed as fold change with respect to untreated Me-CA. These results are a validation of similar results obtained by a multiplex PCR redox gene array analysis (not shown). **(E)** Total antioxidant capacity measured in cell lysates after 24 h of As_2O_3 treatment. In each cell line, data are expressed as % of μM Trolox equivalent of As_2O_3 treated cells with respect to that of untreated control cells (-). Mean ± SD of data from two experiments are shown. Significant differences between As_2O_3-treated group with respect to untreated (-) are indicated; $^*p < 0.05$; $^{**}p < 0.01$. **(F)** Cell surface free thiols in Me-CA cells, untreated or treated 1 h with different amounts of DTT as indicated, were quantified by staining with AFM fluorescent dye and flow cytometry analysis. A representative experiment out of three performed is shown.

increased (Fig. 5C), while thioredoxin reductase, the only known enzyme able to reduce thioredoxin (28), was induced in all cell lines, reaching a very high level in Me-PA (Fig. 5D).

We then analyzed the total antioxidant capacity of the three cell lines before and after exposure to As_2O_3 as a functional index of antioxidant strength (Fig. 5E). While the untreated cell lines displayed a similar total antioxidant capacity, the antioxidant potential was slightly decreased in cells surviving to 24 h As_2O_3 treatment at a comparable extent in the three cell lines. This result suggests that the strong upregulation of the antioxidant systems is sufficient to maintain the intracellular redox homeostasis but does not result in increased functional reducing capacity. This is in contrast to what happens extracellularly, where the redox potential of the medium is functionally reduced by As_2O_3 surviving cells that increase the activity of the cystine/cysteine cycle, resulting in increased extracellular amounts of reduced cysteine (Fig. 4F). The increase in extracellular cysteine is likely to modify the redox status of exofacial thiols, which in turn affects cell behavior (45). Indeed, when melanoma cells are cultured 1 h in culture medium supplemented by 70 μM of DTT, an increase in cell surface protein-free thiols is detected (Fig. 5F). Higher doses of DTT further increase the amount of membrane free sulfhydryls (Fig. 5F). In contrast, treatment with As_2O_3 did not significantly modify the redox state of membrane proteins (not shown), likely due to the obvious dilution and oxidation of the externalized cysteine in the culture medium.

As_2O_3-resistant Me-CA cells maintain an increased rate of cysteine release

Me-CA cells surviving to 48 h of As_2O_3 treatment recovered and regained the proliferative rate of untreated cells within 3 days of culture (data not shown). However, the antioxidant phenotype induced by As_2O_3, with increased cysteine release (Fig. 6A) and thioredoxin expression (Fig. 6B), was maintained in surviving cells after removal of the drug. Also a short exposure to As_2O_3 (6 h), which does not affect cell viability (not shown), upregulated the cystine/cysteine cycle, resulting in higher cysteine release, maintained for at least 48 h (Fig. 6C).

Cells surviving to As_2O_3 treatment further increased the extracellular release of cysteine in response to a new administration of As_2O_3, at a higher extent than non-pre-treated cells (Fig. 6D). Remarkably, at 5 μM As_2O_3, pre-treated cells still increased cysteine release, while non-pre-treated cells decreased it. It is conceivable that this dose (5 μM) of As_2O_3 is toxic for non-pre-treated cells, while pre-treated cells are more resistant and can still upregulate the antioxidant defences. This interpretation was confirmed by the low but significant increase in survival shown by pre-treated cells after exposure to 5 μM As_2O_3 with respect to non-pre-treated cells (Fig. 6E).

Association with redox active drugs enhances the cytotoxic activity of As_2O_3 on melanoma cells

Cells were exposed to various concentrations of As_2O_3 in the absence or presence of BSO or sCPG for 2 days. As shown in Figure 7, the sensitivity to As_2O_3 was increased by the association with BSO in all cell lines, and by the association with sCPG in Me-CA (Panel A) and Me-PA (Panel B). Namely, BSO caused a decrease of As_2O_3 IC50 from 4.3 to 1.6 μM in Me-CA and from 15 to 2.7 μM in Me-PA. The decrease of As_2O_3 IC50 induced by sCPG was less sharp (2.9 μM in Me-CA, 4.2 μM in Me-PA). In the As_2O_3-resistant Me-MOR cells, sCPG did not change the sensitivity to As_2O_3, while the IC50 was decreased by BSO to 3.2 μM (Panel C).

In order to assess whether the response to As_2O_3 observed was shared by other melanoma cells, two additional

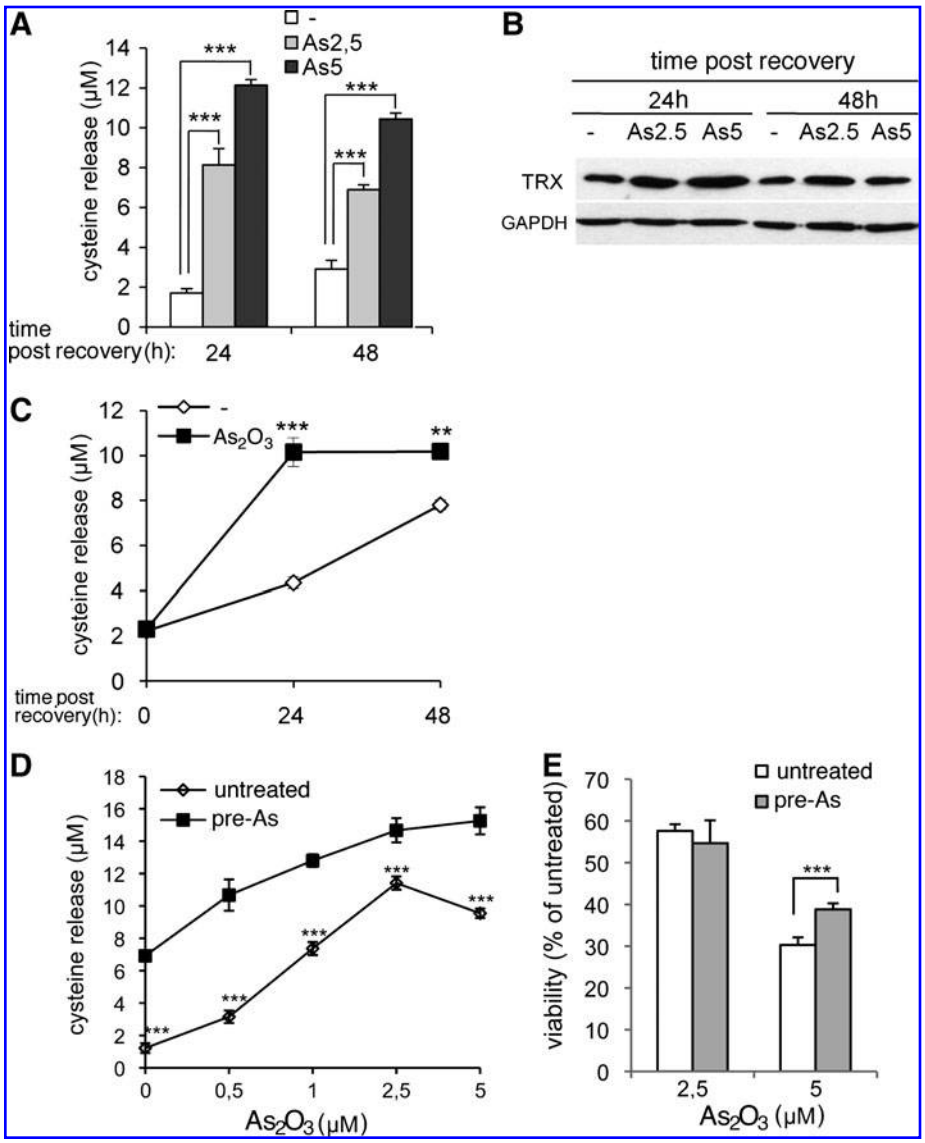

FIG. 6. The increased rate of cysteine release is maintained by As_2O_3-resistant cells. (A, B) Cysteine release **(A)** and Western blot analysis of TRX in cell lysates **(B)** were analyzed 24 or 48 h after recovery in As_2O_3-free medium of Me-CA cells surviving at 48 h treatment with 2.5 or 5 μM As_2O_3. Significant differences between As_2O_3 pre-treated (As2.5 or As5) with respect to untreated (-) cells at each time post recovery are indicated. **(C)** Cysteine release was measured at 24 or 48 h after recovery in As_2O_3-free medium of Me-CA cells pre-incubated 6 h in the absence (-) or presence of 2.5 μM As_2O_3 (As_2O_3). Time 0: cysteine released during the 6 h of incubation with or without As_2O_3 before recovery. Data are expressed as in legend to Figure 1D. Significant differences between As_2O_3 pre-treated (As_2O_3) with respect to untreated (-) cells at each time post recovery are indicated. **(D, E)** Me-CA cells, pre-treated (pre-As) or not (untreated) with 2.5 μM As_2O_3 for 24 h were replated and exposed to different doses of As_2O_3 as indicated. Cysteine release **(D)** and viability **(E)** were assessed after 48 h. Significant differences between As_2O_3 pre-treated (pre-As) cells with respect to untreated cells are indicated at each As_2O_3 dose; $^{***}p < 0.001$; $^{**}p < 0.01$.

melanoma cell lines, Me-OL (5) and SK-Mel cells were subjected to As_2O_3 treatment, in the presence or absence of redox-directed drugs, and antioxidant response and cell survival were evaluated. In both cell lines, As_2O_3 induced a strong upregulation of the cystine/cysteine cycle (Supplementary Figs. S2A and S2B) in the absence of a remarkable increase in GSH (Supplementary Figs. S2C and S2D). Moreover, the sensitivity to As_2O_3 was strongly increased by the concomitant treatment with BSO, and, to a lesser extent, of sCPG (Supplementary Figs. S2E and S2F). These data confirm that the cystine/cysteine cycle is a major antioxidant defense mechanism in As_2O_3-treated melanoma cells, and that redox-directed agents increase the cytotoxic effect of the drug.

Interestingly, also the cytotoxicity of DTIC, a chemotherapeutic agent commonly used in the therapy of malignant melanoma (35), was significantly increased by BSO and sCPG (Figs. 7D and 7E). In addition, DTIC, like As_2O_3 (see Fig. 4F), induced free thiol release (Fig. 7F), indicating that the induction of the cystine/cysteine cycle was not restricted to As_2O_3 treatment but caused also by other anti-melanoma drugs.

Thioredoxin silencing in Me-MOR cells increases the sensitivity to As_2O_3 alone and in combination with sCPG

The three melanoma cell lines differ in the expression of thioredoxin, which is much higher in Me-MOR than in the two other melanoma cases both under basal conditions (Fig. 1E) and following As_2O_3 treatment (Fig. 4E). To investigate whether the lack of effects of sCPG in Me-MOR cells (Fig. 7C) was linked to the strong expression of thioredoxin in these cells, downmodulation of thioredoxin was obtained by transfection with specific siRNA (Fig. 7G). As shown in Figure 7H, 96 h after silencing, no difference in the survival of cells untreated or treated for the last 48 h with sCPG was observed. However, thioredoxin silencing increased the sensitivity to As_2O_3, and, to a higher extent, to the association of sCPG and As_2O_3.

Effects of redox active drugs on the selenium salt selenite

In contrast with the aforementioned observations that melanoma cells that release abundant cysteine are also resis-

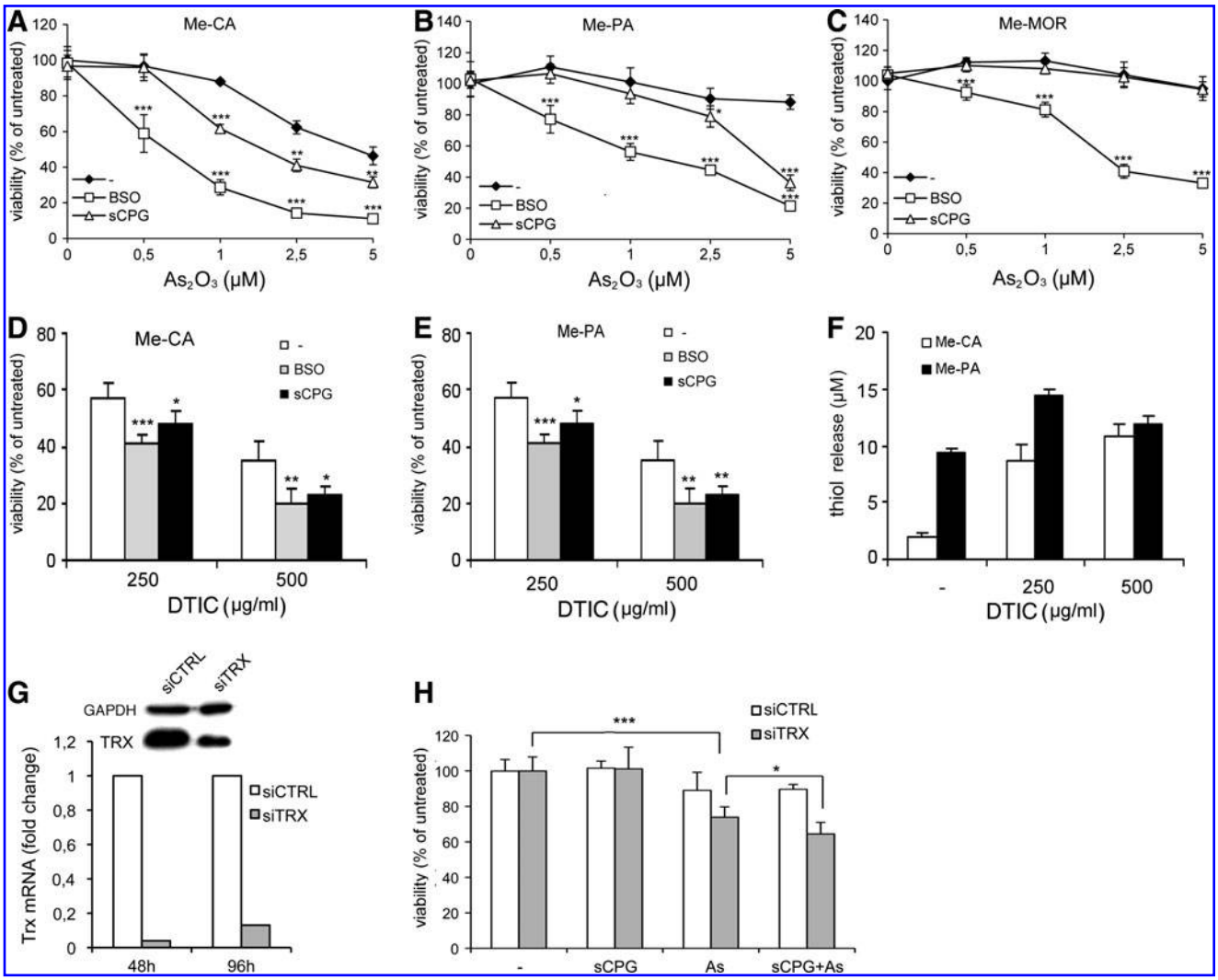

FIG. 7. Either BSO or sCPG increase the melanoma cell responsiveness to As_2O_3 . (A–C) Dose-survival curves of Me-CA **(A)**, Me-PA **(B)**, and Me-MOR **(C)** after 48 h of treatment with As_2O_3 alone (-) or in combination with 100 μM BSO or 100 μM sCPG, as indicated. Data are expressed as percent of viable treated cells with respect to untreated cells. Representative results from one of two independent experiments, each performed with six replicates are shown. At each As_2O_3 dose, significant differences between BSO or sCPG co-treatment with respect to As_2O_3 alone (-) are indicated; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. **(D, E)** Cell viability of Me-CA **(D)** and Me-PA **(E)** after 48 h of treatment with 250 or 500 μg/ml DTIC in the absence or presence of 100 μM BSO or 100 μM sCPG**.** Data are expressed as percent of viable treated cells with respect to untreated cells. Representative results from one out of two independent experiments, each performed with six replicates, are shown. At each DTIC dose, significant differences between BSO or sCPG co-treatment with respect to DTIC alone (-) are indicated; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. **(F)** Extracellular thiols after 48 h of treatment with DTIC were quantified by DTNB assay. Data are expressed as μM. Representative results from one of two independent experiments are shown as the mean of triplicates ± SD. **(G)** Me-MOR cells were transiently silenced with thioredoxin (siTRX) or control (siCTRL) siRNA. Thioredoxin mRNA expression was evaluated after 48 h and 96 h from silencing. *Inset:* Western blot analysis of thioredoxin protein (TRX) content in cell lysates at 96 h from silencing. **(H)** At 48 h from silencing siTRX and siCTRL Me-MOR cells were exposed to sCPG (100 μM), As_2O_3 (5 μM), alone or in combination (sCPG + As) and vitality was evaluated after 48 h by Crystal Violet. Significant differences are indicated; $*p < 0.05$; $***p < 0.001$.

tant to As_2O_3, it has been recently shown that increased activity of the cystine/cysteine cycle correlates with selenite cytotoxicity (38). To investigate whether this positive correlation occurs also in melanoma cells, Me-CA and Me-PA cells, which display the lowest and the highest cystine/cysteine cycle activity, were treated with sodium selenite in the absence or presence of BSO or sCPG. As shown in Figure 8, while BSO slightly increases the selenite toxicity in both Me-CA (Panel A) and ME-PA (Panel B), sCPG suppresses the toxicity of the drug in both cell lines. This inhibitory effect is more evident in Me-PA, since these cells display a higher sensitivity to the drug, in agreement with their higher activity of cystine/cysteine cycle under basal conditions.

Discussion

The redox state and response in tumors is highly complex, and this complexity is further increased by the fact that

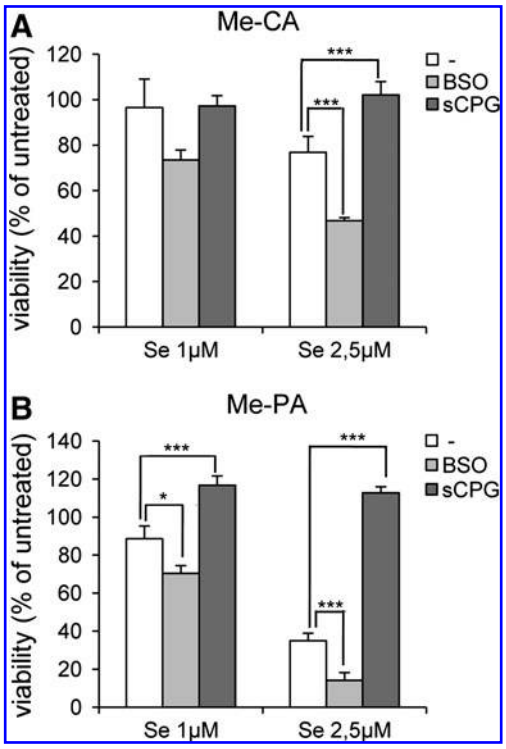

FIG. 8. Effects of BSO or sCPG on sodium selenite-mediated cytotoxicity. Cell viability evaluated in Me-CA **(A)** and Me-PA **(B)** after 48 h treatment with 1 or 2.5 μM sodium selenite (Se) alone (-) or in combination with 100 μM BSO or 100 μM sCPG. Significant differences between BSO or sCPG co-treatment with respect to selenite alone (-) are indicated; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

individual tumors, even among the same histotype, can be characterized by different redox-based signaling mechanisms (16). Thereby, the definition of the adaptive mechanisms operating in different tumors, and of their role in chemoresistance, has tremendous application potential in the development of rational combinations of molecular-targeted redox-active drugs with chemotherapeutics.

This is particularly relevant to malignant melanomas, the most aggressive form of skin cancer (35) whose incidence has increased worldwide over the last 50 years. Indeed, despite the fact that many new chemotherapeutic drugs have been developed and many aggressive treatment options are available, the overall survival has not been significantly improved because most patients with metastatic melanoma are refractory to standard treatments (35). A strong mechanism of chemoresistance in melanoma is represented by the upregulation of antioxidant systems, basal or induced by chemoterapeutic drugs (15, 55).

In this study, we have investigated the prevailing antioxidant mechanisms in melanoma cells from three different melanoma patients and identified two major antioxidant mechanisms, GSH and the cystine/cysteine cycle, shared by the three cell lines although with different degrees.

GSH is a major antioxidant system, both in healthy and neoplastic cells, and sustenance of GSH levels through GSH biosynthesis is vital for growth and survival of tumors (34, 58). The xCT transporter is recognized as an important player in cancer (30), but how the cystine/cysteine cycle contributes to tumor progression is still controversial. Most studies proposed that xCT functions as a supplier of cystine for GSH synthesis (12, 36). Increased cellular uptake of cystine would then contribute to drug resistance in ovarian (37), lung (18), and pancreatic cancer cells (29) by enhancing biosynthesis of glutathione. In contrast, here we show that in melanoma cells the two mechanisms are independent, since only a small portion of the internalized cystine is used for GSH synthesis, while most of it is recycled outside cells as reduced cysteine, resulting in a reduction of the extracellular redox state. These results are in agreement with the observation that overexpression of xCT in lymphoma cells confers resistance to oxidative stress in the condition of GSH depletion (3, 32), and indicate that the autonomous antioxidant function of xCT is not restricted to hemopoietic cells. Interestingly, while melanoma cells are resistant to either GSH depleting drugs or compounds blocking the xCT activity, the simultaneous targeting of the two antioxidant systems rapidly leads to cell death, indicating that the two systems may substitute for each other but cannot be replaced with other antioxidant systems. Besides identifying the two major antioxidant systems in melanoma cells, these findings sort out the complexity of the redox repertoire in melanoma cells and provide well-defined targets for redox-modulating therapies.

Arsenic elicits its toxic effects through different mechanisms, including generation of oxidative stress. As_2O_3-induced oxidative stress triggers an Nrf2-dependent antioxidant response (33). In agreement, we observed that a number of antioxidant genes, including xCT and the members of the GSH and thioredoxin systems, are upregulated in As_2O_3-treated melanoma cells. Remarkably, however, in all the three melanoma cases the redox remodeling consequent to exposure to sublethal doses of As_2O_3 has, as a net effect, a strong reduction of the extracellular redox state whereas the intracellular redox state remains essentially balanced. Indeed, intracellular GSH and total antioxidant capacity were substantially unaffected in cells surviving As_2O_3 treatment. This behavior is only apparently incoherent. In fact, antioxidant systems, already overexpressed in untreated neoplastic cells, may buffer the As_2O_3-induced ROS, resulting in failure to detect increased ROS levels. Consistent with these findings, it has been recently reported that As_2O_3 induces ROS in nontransformed cells that display physiologic levels of antioxidants but not in transformed cells, that overexpress antioxidant systems (9). This buffering mechanism, however, causes an unbalance in redox homeostasis, which is corrected by further upregulation of antioxidant systems. In turn, the increase of antioxidant system activity is sufficient to restore the intracellular redox balance but not to induce a net increase of intracellular antioxidants. In contrast, due to the high amounts of cysteine externalized upon upregulation of the cystine/cysteine cycle, the redox state of the extracellular microenvironment results strongly reduced. Interestingly, DTIC, a common anti-melanoma drug, elicits a redox remodeling similar to that induced by As_2O_3, suggesting that these effects can be obtained also with other antineoplastic compounds.

The switch of the extracellular redox from a physiologic oxidizing state to a reduced state may promote tumor progression through various mechanisms. Emerging data indicate that cell surface protein thiols are targets of redox regulation and that the redox status of such thiols controls

critical cellular functions (8). Therefore, a major effect of the reduced extracellular redox may be the modulation of exofacial thiols of membrane proteins, which may lead to an altered signaling, promoting tumor progression. In support to this hypothesis, the anti-tumor compound parthenolide, endowed with nucleophilic properties, has been found to exert its cytotoxic activity on lymphoma and melanoma cells by attenuating the levels of exofacial free thiols (27, 45). In our cell system, a well-detectable increase in the amount of membrane-bound free thiols is induced by the addition of 70 μM DTT to culture medium. This concentration of antioxidants is not reached in As_2O_3-treated melanoma cell supernatants *in vitro* (due to dilution and oxidation of the externalized cysteine in culture medium) but it is conceivably easily achieved by cysteine-releasing melanoma cells *in vivo* in the tumor microenvironment. The reduced extracellular redox state may also influence half-life and activity of soluble proteins, such as HMGB1, a damage-associated molecular pattern molecule (DAMPs) which has been implicated in tumor progression (47). HMGB1 is highly expressed by melanoma cells (not shown) and is released by As_2O_3-killed melanoma cells (Fig. 3). Due to the cytosolic reducing environment, cysteine residues in intracellular HMGB1 are reduced. When released in the oxidizing extracellular milieu, HMGB1, like other redox-sensitive DAMPs, undergoes oxidation with modification of its function (41). However, the presence of a reducing extracellular microenvironment with high levels of cysteine released by melanoma cells surviving As_2O_3 treatment may delay oxidation, causing a prevalence of reduced vs. oxidized HMGB1 at the tumor site. The reduced state may favor some extracellular activities of the molecule, such as induction of cell proliferation, over others such as immunostimulation, with potential promotion of tumor progression (47).

As_2O_3 treatment can worsen the course of the neoplastic disease also by inducing in As_2O_3-resistant tumor cells a transiently reduced phenotype that make them able to survive higher doses of the drug. Since tumors overexpressing antioxidant systems are more aggressive and exhibit a poorer prognosis (39, 46, 58), the final effects of pro-oxidant therapies may be the selection of melanoma cell clones that not only are intrinsically more malignant, but may also benefit from the extracellular reduced environment. Therefore, the association with redox-active drugs is not only useful to increase the toxicity of pro-oxidant compounds but also to avoid tumor progression accomplished by surviving cells. In this connection, while BSO potentiates As_2O_3 in all the cell lines, being in principle the redox-active drug of choice for association therapies with As_2O_3, the use of sCPG or other blocker of cystine/cysteine cycle is recommended, since it prevents the reduction of the extracellular redox state and, consequently, the possibly detrimental effects this alteration plays on tumor progression.

Finally, we have shown that, in sharp contrast with the results obtained with As_2O_3, sCPG completed reverted the toxicity of selenite on the melanoma cells. This result confirms and extends the observation that a reducing microenvironment, dependent on the activity of the cystine/cysteine cycle, leads to a high-affinity selenium uptake by cancer cells, and consequently increases selenite cytotoxicity (38). The highest sensitivity to selenite was displayed by Me-PA cells that release more cysteine than the other cell lines under basal conditions. Therefore, selenite may represent a treatment of choice for those tumors where the cystine/cysteine cycle causing chemoresistance towards common anti-tumor drugs is so expanded that it cannot be overcome using association with redox-directed drugs.

Immunostaining of tumor samples with anti xCT antibodies may represent a novel and easy way to distinguish tumors with low or high expression of xCT, as a marker of sensitivity to different chemotherapeutics. While tumors weakly stained for xCT are likely responsive to treatment with pro-oxidant compounds, tumors overexpressing xCT, most likely resistant to pro-oxidants, may benefit from the treatment with selenite.

In conclusion, we have shown that GSH and the cystine/cysteine cycle have a crucial role in redox metabolism of melanoma cells, and that extracellular redox state, more than the intracellular one, is remodeled and strongly reduced following As_2O_3 treatment. We have also demonstrated that the selective targeting of these systems improve the effectiveness of pro-oxidant chemotherapeutics such as As_2O_3, and prevent the generation of a disturbed microenvironmental redox state responsible for abnormal behavior of cancer or neighboring cells (8).

Materials and Methods

Chemicals

Arsenic trioxide (As_2O_3), buthioninesulfoximine (BSO), dacarbazine (DTIC), sodium selenite, 5-5′-dithio-bis(2-nitrobenzoic acid) (DTNB), dithiothreitol (DTT), Mercury Orange, *N*-ethylmaleimide were purchased from Sigma-Aldrich. (*S*)-4-carboxyphenylglycine (s-CPG) was purchased from Tocris. AlexaFluor 488-C5 maleimide (AFM), 2′,7′-dichlorofluorescein diacetate (H_2DCF-DA), and monochlorobimane (MCB) were obtained from Molecular Probes.

Staining procedures and immunohistochemistry

Serial cryostat sections of frozen sample from human melanoma metastases were stained with the sulfhydryl-reactive dye Mercury Orange as previous described (7, 52). Briefly, Mercury Orange was incubated 5 min on ice at a final concentration of 25 μM. To confirm staining specificity to nonprotein thiols (GSH and cysteine), control sections were pretreated with 100 μM *N*-ethylmaleimide for 10 min to block thiol groups. The sections were then analyzed by confocal microscopy, and the images were acquired with the Fluoview FV500 software.

For immunohistochemical analyses, sections were stained by rabbit anti-human xCT antibody (Novus Biologicals), followed by a streptavidin–biotin–alkaline phosphatase-complex staining kit (Bio-SPA Division). For each melanoma case, three representative zones were sampled. Images were acquired with Leica DM LB-2 microscopy using Scion Image software (7).

Cell cultures

Malignant melanocytes obtained from surgical specimens of skin metastatic melanoma from three patients were adapted to grow *in vitro* in RPMI supplemented with 10% FBS, glutamine, and penicillin/streptamycin (Sigma-Aldrich) (5).

After adhesion, cells were cultured in RPMI 5% FBS. After 3–5 passages, cells were analyzed by immunocytochemistry for the expression of melanocytic (anti-Vimentin monoclonal Ab, Dako) and malignant melanoma (anti-S-100, anti-HMB45 and anti-Mart-1 monoclonal Ab, Ventana Medical Systems) cell markers. Of the three cell lines (Me-CA, Me-PA, and Me-MOR), Me-CA and Me-PA were strongly positive for the four markers. In contrast, Me-MOR was positive for vimentin only, in spite of the histologic and clinical diagnosis of malignant melanoma. The phenotype of the three cell lines was stable and maintained for the duration of the experiments described in this paper. In addition, the human melanoma cell lines Me-OL (5) and SK-MEL-28 (obtained from ICLC Interlab Cell Line Collection, Genova, Italy) were also used in selected experiments.

ROS and GSH detection

Intracellular ROS and reduced GSH content were evaluated as described in (49, 52). Briefly, cells were incubated for 30 min at 37°C with 10 μM H_2DCFDA to assess intracellular ROS or 100 μM MCB to assess intracellular GSH. Fluorescence intensity was measured in cell lysates by spectrofluorimetry (excitation 480 nm and emission 530 nm for H_2DCFDA; excitation 355 nm and emission at 485 nm for MCB) or on whole cells by flow cytometry (CyAn, Beckman Coulter), followed by Summit V4.3 software analysis. Protein content from each sample, evaluated by DC protein assay (Bio-Rad) was used to normalize fluorescence signal intensity. In selected experiments, total or reduced form of GSH were measured by a commercial kit based on the GSH recycling system by DTNB and GSH reductase (MBL), according to the manufacturer instructions.

Total antioxidant capacity

Total antioxidant capacity in cell lysates were performed using the commercial kit from Cayman, following manufacturer instructions with small modifications. Briefly, cells were washed three times with PBS, scraped, and lysed. Protein content was evaluated and 20 μg of proteins were tested. The Trolox equivalent antioxidant activity was measured by assessing the ability of hydrogen-donating antioxidants to scavenge the radical cations generated by 2,2-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid (40). Data were calculated as μM Trolox equivalent per μg protein.

Determination of free thiol release in culture media

Cell supernatants were reacted with 10 mM DTNB and the absorption measured at 412 nm, as previously described (7, 49, 52). To discriminate between extracellular cysteine and GSH, S-carboxymethyl derivatives of soluble thiols were generated as described (49) and NPSHs were separated by HPLC with a Bondapak NH2 column (Warters). GSH, oxidized glutathione (Roche), cystine, and cysteine (Sigma-Aldrich) were used as external standards.

Western blot analysis

Aliquots (30 μg) from total cell lysate proteins were resolved on 10% or 12% SDS-PAGE, and electrotransferred. Membranes were probed with anti-human Trx monoclonal Ab (clone 2B1, kind gift of Dr. F. Clarke, Brisbane, Australia), rabbit anti-human xCT antibody (Novus Biologicals), or anti GAPDH (Novus Biologicals), followed by the relevant secondary antibody (DAKO) and developed with ECL-plus (GE Healthcare), as described (7).

Cell surface free thiol measurements

Melanoma cells were cultured 1 h in the absence or presence of increasing amounts of DTT (from 30 to 1000 μM). Free surface thiol groups were detected using the AlexaFluor 488-C5 maleimide (AFM). Briefly, cells were incubated with 10 μM AFM for 30 min at 37°C and fluorescence was measured by a flow cytometer (CyAn, Beckman Coulter) and analyzed using Summit V4.3 software (45).

Detection of HMGB1 in cell supernatants

Supernatants from cells treated in RPMI supplemented with Nutridoma-SP (Roche) were collected and concentrated by 10% trichloroacetic acid precipitation as previously described (43). The presence of HMGB1 was detected by Western blotting with anti-HMGB1 antibody (kind gift of Dr. MT Lotze, Pittsburgh, PA).

Cell viability and cytotoxicity assays

Cell proliferation and viability were determined by the crystal violet assay and cytotoxicity was measured as release of lactate dehydrogenase (LDH) into the culture medium using the colorimetric CytoTox 96 assay kit (Promega) as previously describes (53).

Real-time PCR

Total RNA was isolated using TriPure Isolation Reagent (Roche) and reverse-transcribed by QuantiTect Reverse Transcription kit (Qiagen), according to manufacturer's instructions. Real-time PCR determination of cDNA was performed using Platinum SYBR Green qPCR Super Mix -UDG (Invitrogen). The specific primers used were: forward 5′gctgggctgatttatcttcg3′ and reverse 5′aaagctgggatgaaca gtgg3′ (for xCT); forward 5′agcagatcgagagcaagact3′ and reverse 5′cactct gaagcaacatcctg3′ (for thioredoxin), forward 5′tgaagagaggcatgttggag3′ and reverse 5′atgatgcaatggtctcct ga3′ (for SOD1); forward 5′cccgatgtatcacgcagtta3′ and reverse 5′ttcactgcaacagcaaaacc3′ (for GSH reductase); forward 5′cttgtggcctttctgaggag3′ and reverse 5′ctcttgacggaatcgtccat3′ (for Thioredoxin reductase-1); forward 5′gaaggtgaaggtcg gagtc3′ and reverse 5′catgggtggaatcatattggaa3′ (for GAPDH). The levels of mRNA expression were normalized with the expression of the GAPDH housekeeping gene. Relative expression was determined using the ΔΔCt method (48, 52).

To determine gene expression related to oxidative stress, a focused pathway RT^2 Profiler PCR Arrays was used. cDNA was prepared and analyzed by Human defense oxidative stress and antioxidant defense PCR array (PAH-S065A SABioscences), according to the manufacturer's instructions.

Thioredoxin silencing

Me-MOR cells were transfected with 10 nM of thioredoxin siRNA (Invitrogen) using Lipofectamine™ RNAiMAX reagent (Invitrogen) according to manufacturer's

recommendations (7). A dsRNA sequence with no homology to the human genome (Stealth RNAi Negative Control Duplexes-Invitrogen) has been used as negative control. After 48 h and 96 h from silencing, thioredoxin expression was evaluated by real-time PCR and western blotting.

Statistical analysis

The data were statistically analyzed by unpaired two tailed *t*-test or by one-way ANOVA test, followed by Bonferroni post-test using Excel or GraphPad software.

Acknowledgments

We thank Drs. F. Clarke and M.T. Lotze for the generous gift of antibodies, Dr. G. Ferlazzo for kindly providing the melanoma cell lines, and Dr. A. Omenetti for editing the manuscript. We also thank CRB-IST (www.istge.it/crb/index.htm) for the supply of human melanomas samples. This work was supported in part by grants from Ministero della Salute, ISS, Compagnia di San Paolo, Telethon.

Author Disclosure Statement

No competing financial interests exist.

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Date of first submission to ARS Central, December 7, 2010; date of final revised submission, April 6, 2011; date of acceptance, April 29, 2011.

Abbreviations Used

As_2O_3 = arsenic trioxide
BSO = buthioninesulfoximine
DAMPs = damage associated molecular pattern molecules
DTIC = dacarbazine
DTNB = 5-5′′-dithio-bis(2-nitrobenzoic acid)
DTT = dithiothreitol
GSH = glutathione
H_2DCF-DA = 2′,7′′-dichlorodihydrofluorescein diacetate
HMGB1 = High Mobility Group Box 1
MCB = monochlorobimane
RFU = relative fluorescence units
ROS = reactive oxygen species
sCPG = (S)-4-carboxyphenylglycine
SOD-1 = superoxide dismutase 1

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