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Glutathione Influences c-Myc-induced Apoptosis in M14 Human Melanoma Cells*

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The objective of this article is to dissect the mechanisms by which the down-regulation of c-Myc induces programmed cell death in melanoma cells. In stable and doxycycline-inducible M14 melanoma cells, down-regulation of c-Myc induced apoptosis subsequent to a decrease in the intracellular reduced glutathione content and a concomitant accumulation of its oxidized form. This redox alteration was associated with a decrease of the enzyme activities of γ-glutamyl-cysteine synthetase and NADPH-dependent GSSG reductase, as well as a consequent glutathione release in the extracellular medium. Cytochrome *c* was released into the cytosol at very early stages of apoptosis induction, long before detectable production of reactive oxygen species and activation of caspase-9 and -3. Macroarray analysis revealed that down-regulation of c-Myc produced striking changes in gene expression in the section related to metabolism, where the expression of γ-glutamyl-cysteine synthetase and GSSG reductase was found to be significantly reduced. The addition of *N*-acetyl-L-cysteine or glutathione ethyl ester inhibited the apoptotic process, thus confirming the key role of glutathione in programmed cell death induced by c-Myc.

Maintenance of normal function of cells and tissues is dependent on precise regulation of multiple signaling pathways that control cellular decisions to either proliferate, differentiate, arrest cell growth, or initiate programmed cell death. Genes of the *myc* family, including c-*myc*, have been implicated in the regulation of many cellular processes such as proliferation, differentiation, and transformation (1, 2). Deregulated expression of c-*myc* accelerates apoptosis in myeloid cells (interleukin-3-dependent) deprived of interleukin-3 (3) and in serum-deprived fibroblasts (4). Over-expression of c-*myc* induced apoptosis under certain conditions such as viral infection and treatment with tumor necrosis factors and chemotherapeutic agents (5–7). Despite intensive research, the molecular mechanisms underlying apoptosis mediated by c-Myc are yet to be understood. It has been proposed that c-Myc induces apoptosis by up-regulating its target genes, such as those expressing ornitine decarboxylase (8, 9), lactate dehydrogenase (10, 11), cyclin A, cdc25A (12, 13), or p53 (14, 15). Moreover, at least in tumor cells, not only the over-expression of c-*myc* but also its down-regulation induces apoptosis (16–18). In this context, we previously demonstrated that treatment with c-*myc* antisense oligodeoxynucleotides caused a significant inhibition of cell proliferation and induced apoptosis in several human melanoma cell lines (19–21). By using stable transfectants, we also demonstrated that apoptosis following down-regulation of c-Myc (22) was associated with an increase in the production of ROS[1] (23). In fact, oxidative stress is a well known inducer of apoptosis (24, 25). In particular, it has been demonstrated extensively that apoptosis is stimulated by cell depletion of GSH (26, 27), the low molecular weight thiol that is crucial for antioxidant defense (28). In this context we demonstrated that following apoptogenic stimuli that do not directly elicit an oxidative stress, GSH is extruded from the cells at very early stages of the process (29) and that the inhibition of the extrusion of GSH prevents apoptosis (30). Moreover, we evidenced that the release of cytochrome *c* from mitochondria followed cell depletion of GSH, independently of the destiny of the cells, *i.e.* apoptosis or survival (31).

The present study aims at dissecting the molecular mechanism by which down-regulation of c-Myc induces apoptosis, with the expectation of identifying new molecular factors that may represent therapeutic targets in reestablishing apoptotic pathways in cancer cells. We demonstrate that down-regulation of c-Myc triggers apoptosis in stable and doxycycline-inducible clones of M14 melanoma cells through a canonical redox-mediated pathway involving depletion of GSH and release of cytochrome *c* as early events, whereas production of ROS and activation of caspases are late effectors. Decrease of GSH results from an impairment of both its synthesis and GSSG reduction. The key role of GSH in the apoptosis induced by down-regulation of c-Myc is further supported by the ability of Cys-NAc or GSH ester to suppress the commitment of cells to death.

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[1] The abbreviation used are: ROS, reactive oxygen species; γ-GCS, γ-glutamyl-cysteine synthetase; PARP, poly(ADP-ribose) polymerase; Cys-NAc, *N*-acetyl-L-cysteine; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); PI, propidium iodide; HPLC, high pressure liquid chromatography.

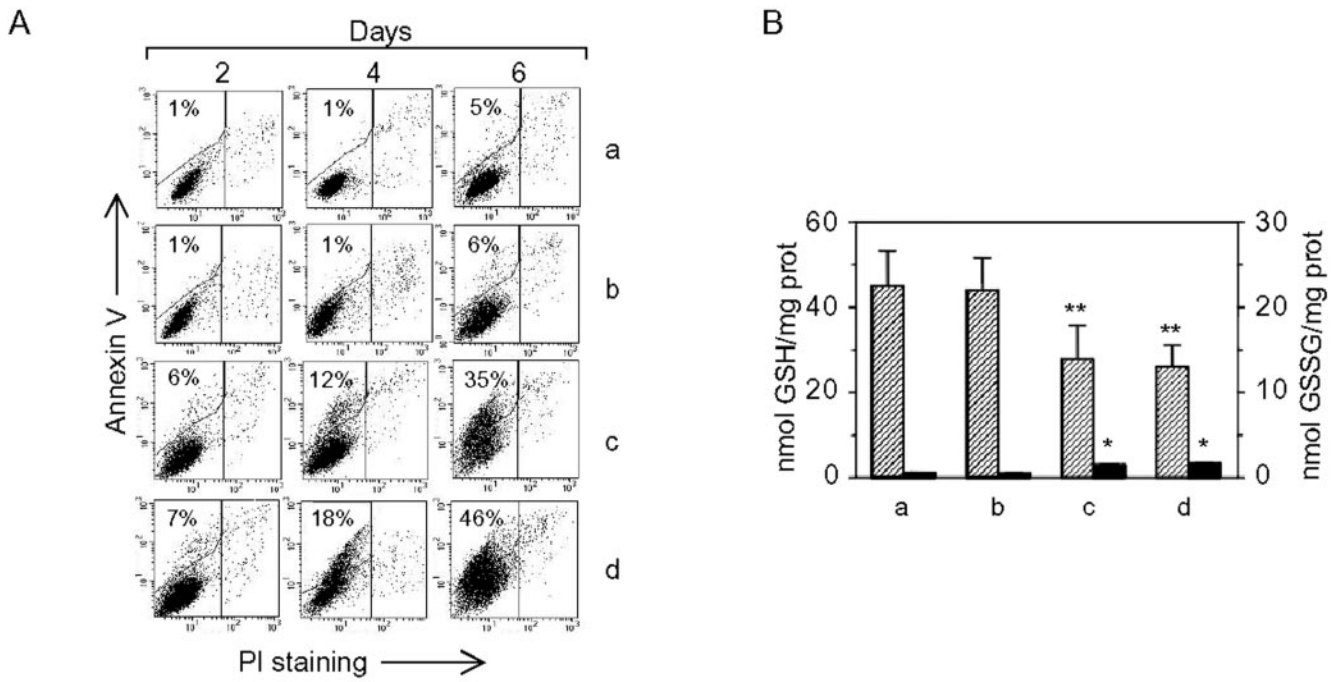

FIG. 1. **Apoptosis induced by down-regulation of c-Myc is associated with cell depletion of GSH.** *A*, cytofluorimetric analysis of the annexin V *versus* PI staining assay performed in M14 (*a*), MN2 (*b*), MAS51 (*c*), and MAS53 (*d*) cell lines on days 2, 4, and 6 of culture; ($n = 5$). The percentage reported in the annexin V+/PI− region of each histogram represents the apoptotic cells. *B*, intracellular GSH (*slashed columns*) and GSSG (*black columns*) content was measured in M14 (*a*), MN2 (*b*), MAS51 (*c*), and MAS53 (*d*) transfectants on day 4 of culture as described under "Experimental Procedures" ($n = 10$); *, $p < 0.05$; **, $p < 0.01$.

EXPERIMENTAL PROCEDURES

Cell lines, Culture Conditions, and Treatments—The stable M14 melanoma transfectants (MAS51 and MAS53 c-Myc low expressing clones and MN2 control clone) were obtained by transfection with an expression vector carrying antisense c-*myc* cDNA and/or a selection marker gene (23). The M14-derived doxycycline-inducible clones expressing low c-Myc (MAS IND1 and MAS IND18) were obtained by a double transfection with a commercial inducible TET-ON gene expression system (Clontech, Florence, Italy) consisting of two expression vectors, a regulator and a response one carrying c-*myc* cDNA (exon 2 + exon 3) in the antisense orientation. Doxycycline (1 μg/ml, administered every 24 h) down-regulates c-Myc protein in about 72 h by 50–60% in both clones as compared with uninduced transfectants.

Materials—DTT, EDTA, EGTA, PIPES disodium salt, potassium borohydride, GSH ethyl ester, L-glutamate, L-α-aminobutyrate, and Cys-NAc were obtained from Sigma. GSH, GSSG, ATP disodium salt, phosphoenolpyruvate, NADH, NADPH, and pyruvate kinase/lactate dehydrogenase were purchased from Roche Molecular Biochemicals. All other chemicals were obtained from Merck.

ROS, Apoptosis, and Caspase Activity—For ROS content, adherent cells were first assayed for viability and then incubated with 4 μM dihydroethidium (DHE, Molecular Probes, Eugene, OR) for 45 min at 37 °C. After incubation, the cells were analyzed by flow cytometry.

Apoptosis was evaluated by annexin V *versus* PI assay. Cells were harvested, suspended in annexin-binding buffer (1×10^6 cells/ml), incubated with fluorescein isothiocyanate-annexin V and PI (Molecular Probes) for 15 min at room temperature in the dark, and then immediately analyzed by flow cytometry. The annexin V positive/PI negative (annexin V+/PI−) cells were considered apoptotic.

Activity levels of caspase-9 and -3 were evaluated using a CaspaTagTM Kit according to the manufacturer's instruction (Intergen, Oxford, UK). Cells were harvested and resuspended in fresh medium (1×10^6 cells/ml). The specific fluorescent caspase substrate was added directly to the cell suspension and left for 1 h at 37 °C under 5% CO_2 and protected from the light. After washing, cells were stained with PI and analyzed by flow cytometry.

Determination of Cytosolic Cytochrome c—Cells were washed with phosphate-buffered saline and collected by centrifugation at $700 \times g$ for 7 min at 4 °C. The cell pellet was resuspended in extraction buffer containing 220 mM mannitol, 68 mM sucrose, 50 mM PIPES-NaOH, pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol, and protease inhibitors. After a 30-min incubation on ice, cells were homogenized with a glass Dounce homogenizer. Cell homogenates were spun at $14,000 \times g$ for 15 min at 4 °C, and supernatants were removed and stored at −80 °C until analysis by gel electrophoresis. 20 μg of cytosolic protein extracts were loaded onto each lane of a 12% SDS-PAGE, separated, and then blotted to nitrocellulose membrane (Bio-Rad). Purified mouse anti-cytochrome *c* antibody (clone 7H8.2c12, Pharmingen) was used as primary antibody (1: 5,000). The specific protein complex, formed upon anti-mouse secondary antibody treatment (1:10,000), was identified using SuperSignal substrate chemiluminescence reagent.

Western Blotting—40 μg of total proteins were loaded from each sample on denaturing 12% SDS-PAGE. Immunodetection of PARP, γ-GCS, c-Myc, and β-actin were performed using rabbit anti-PARP (1:2000, Roche Molecular Biochemicals), rabbit anti-γ-GCS (1:1000; provided by Dr. A. Cantin, Service de pneumologie, CHUS-Fleurimont), mouse anti-c-Myc (1:1000, clone 9E-10, Santa Cruz Biotechnology), and mouse anti-actin (1:1000, clone AC-40, Sigma) antibodies. ECL was used for detection.

Determination of Glutathione—For intracellular glutathione determination, cell monolayers were washed with phosphate-buffered saline, resuspended, and lysed by repeated cycles of freezing and thawing under liquid nitrogen. Lysates were acidified with 5% *meta*-phosphoric acid and centrifuged at $22,300 \times g$ for 15 min at 4 °C. Low molecular weight free thiols were derivatized to *S*-carboxymethyl derivatives upon treatment of supernatants with iodoacetic acid. GSH and GSSG concentrations were determined by the conversion of free amino groups to 2,4-dinitrophenyl derivatives by the reaction with 1-fluoro-2,4-dinitrobenzene. Low molecular weight thiols were, finally, separated by HPLC using a μBondapak NH_2 column (Waters) as described by Reed *et al.* (32). GSH and GSSG were used as external standards. Data were expressed as nmol/mg protein. For the extracellular glutathione assay, culture media were collected and centrifuged at $700 \times g$ for 10 min at 4 °C in order to discard detached cells. Media were then acidified and treated as described before. Data were expressed in nmol/ml.

Macroarray Analysis of Gene Expression—Total RNA was isolated from exponentially growing cells using Trizol reagent (Invitrogen) following standard protocols and quantified spectrophotometrically. Poly(A)$^+$ RNA was extracted from 50 μg of total RNA using the Oligotex mRNA purification system (Qiagen, Milano, Italy) and retrotranscribed to cDNAs in the presence of [^{32}P]dATP (Amersham Biosciences) using a mixture of specific oligonucleotides (Clontech). Equal amounts of labeled cDNAs were hybridized to filters containing 1200 genes (ATLAS

1.2 human Cancer, Clontech). After being washed, the filters were autoradiographed, and gene expression patterns were evaluated using the ATALS Image software (Clontech). Expression intensities were considered comparable only if at least one of the two samples (c-Myc low expressing clone and control clone) had intensity greater than 2-fold the background value. Three macroarrays were performed for MN2 control clone, and the values for each gene were averaged.

Enzyme Activities—γ-GCS was assayed as described by Seelig and Meister (33). The cell pellet was lysed in 0.1 M Tris-HCl, pH 8, containing 5 mM $MgCl_2$ and 2 mM dithiothreitol and centrifuged at 12,550 × *g* for 30 min. Supernantants were used for γ-GCS determination following oxidation of NADH at 340 nm in 0.1 M Tris-HCl, pH 8, containing 150 mM KCl, 5 mM Na_2-ATP, 2 mM phosphoenolpyruvate, 10 mM L-glutamate, 10 mM L-α-aminobutyrate, 20 mM $MgCl_2$, 2 mM Na_2-EDTA, 0.2 mM NADH and 17 μg of pyruvate kinase/lactate dehydrogenase. Data were expressed as nmol of NADH oxidized/mg of protein. GSSG reductase activity was monitored spectrophotometrically, as described previously (34), by following the changes in absorbance at 340 nm due to the oxidation of NADPH and using as substrates 0.17 mM NADPH and 2.2 mM GSSG in 100 mM phosphate buffer, 0.5 mM EDTA, pH 7.2, at 37 °C. Data were expressed as nmol of NADPH oxidized/mg of protein.

Statistical Analysis—The results are presented as means ± S.D. Significant changes were assessed using Student's *t* test for unpaired data, and p values < 0.05 were considered significant.

RESULTS

Apoptosis Mediated by Down-regulation of c-Myc Is Associated with Cell Depletion of GSH—Two M14 melanoma transfectants (MAS51 and MAS53) expressing 6–10 times less c-Myc protein than the control clone (MN2) and M14 parental line (23) were employed. By means of the annexin V *versus* PI staining assay (Fig. 1*A*), we demonstrated that MAS51 and MAS53 clones showed spontaneous apoptosis, which increased from about 6% on day 2 to about 40% on day 6. On the contrary, no apoptosis was observed in either of the control lines for up to 6 days of growth. On day 4 of growth, when about 15% of apoptosis was evident in both MAS51 and MAS53 transfectants, the values of GSH were significantly lower ($p < 0.01$) than both the M14 and MN2 control cells, with a concomitant significant increase ($p < 0.05$) in the GSSG form of the tripeptide, whereas mixed disulfides between glutathione and cysteine residues of proteins were undetectable (Fig. 1*B*). Fig. 2*A* shows that GSH content was significantly different as early as 2 and 3 days of growth (*, $p < 0.05$; **, $p < 0.01$), far ahead of the time when the apoptotic process was activated, indicating that its decrease represents an upstream event in the induction of apoptosis.

The steady state level of GSH in the cell results from a balance between the rates of synthesis and loss of the tripeptide via oxidation or excretion. Under our experimental conditions we detected an increase in intracellular GSSG content as early as 2 days of growth (Fig. 2*B*); this increase is commonly observed when cells are oxidatively stressed. This evidence could, at least in part, provide an explanation for the lower levels of GSH present in MAS51 and MAS53 clones. On the other hand, a significant increase in GSH content was determined in the culture media of these clones (Fig. 2*C*) on days 3 and 4 of growth ($p < 0.01$and 0.001, respectively).

Down-regulation of c-Myc Reduces γ-GCS and GSSG Reductase Expression and Activity Levels—In search of a mechanism responsible for the early alterations observed in GSH content following down-regulation of c-Myc, we monitored two key enzymes determining the balance of glutathione forms in the cells: γ-GCS, which is the rate-limiting enzyme in GSH synthesis, and the NADPH-dependent GSSG reductase, which is responsible for efficient back-reduction of GSSG to GSH. Fig. 3 shows the activity (*panel A*) and protein levels (*panel B*) of γ-GCS. The results show a significant decrease in activity (about 40%) as early as day 2 of growth, with no further

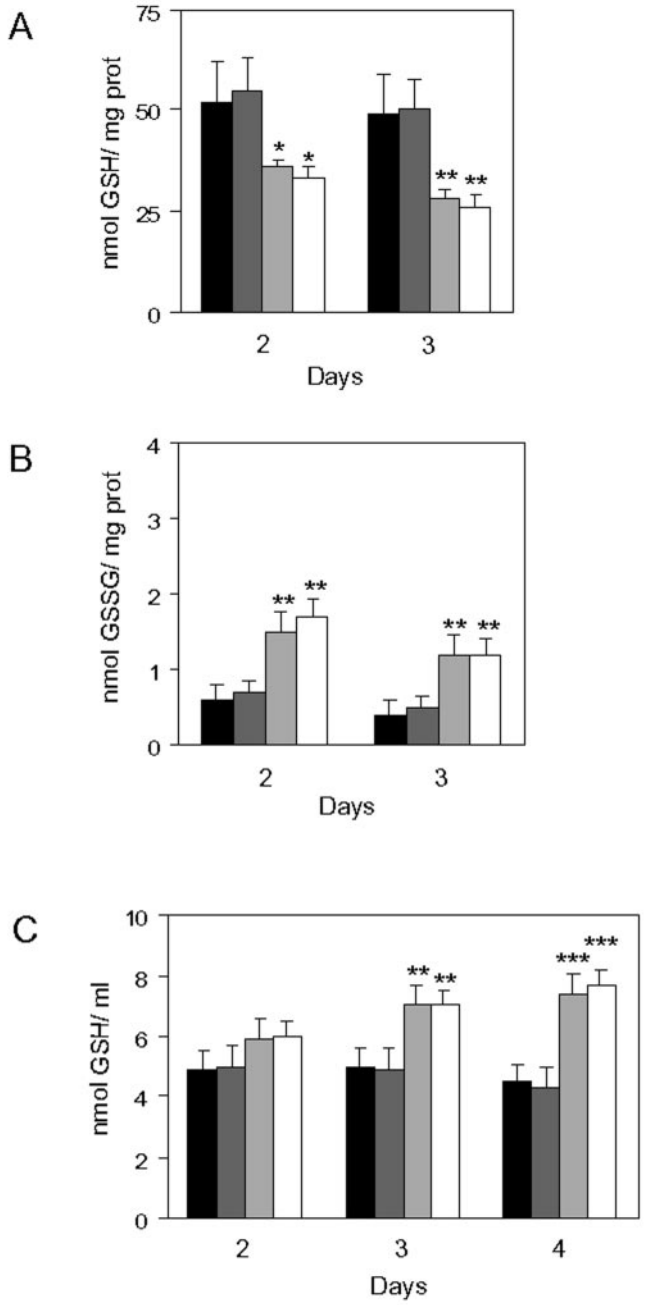

FIG. 2. **The decrease of GSH is an early event in the induction of apoptosis.** HPLC analysis of intracellular GSH (*A*), intracellular GSSG (*B*), and extracellular GSH content (*C*) was performed in M14 (*black columns*), MN2 (*dark gray columns*), MAS51 (*light gray columns*), and MAS53 (*white columns*) cells. On the indicated days, cells and media were treated for glutathione determinations as described under "Experimental Procedures" ($n = 10$); *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

decrement in either the MAS51 or MAS53 transfectant when compared with the MN2 and M14 parental line. These data are in line with the protein concentration of the two subunits of the synthetase, the heavy catalytic (γ-GCS_H) and the light regulatory (γ-GCS_L) subunit, as assayed by Western blot analysis (Fig. 3*B*). Similarly, the activity of GSSG reductase showed a significant decrease in both of the clones expressing low c-Myc (Fig. 3*C*), thus correlating well with the changes in GSSG content observed in the MAS51 and MAS53 transfectants as compared with control lines.

The dependence of gene expression profiles on c-Myc was analyzed by macroarray technology (Fig. 4). The figure shows

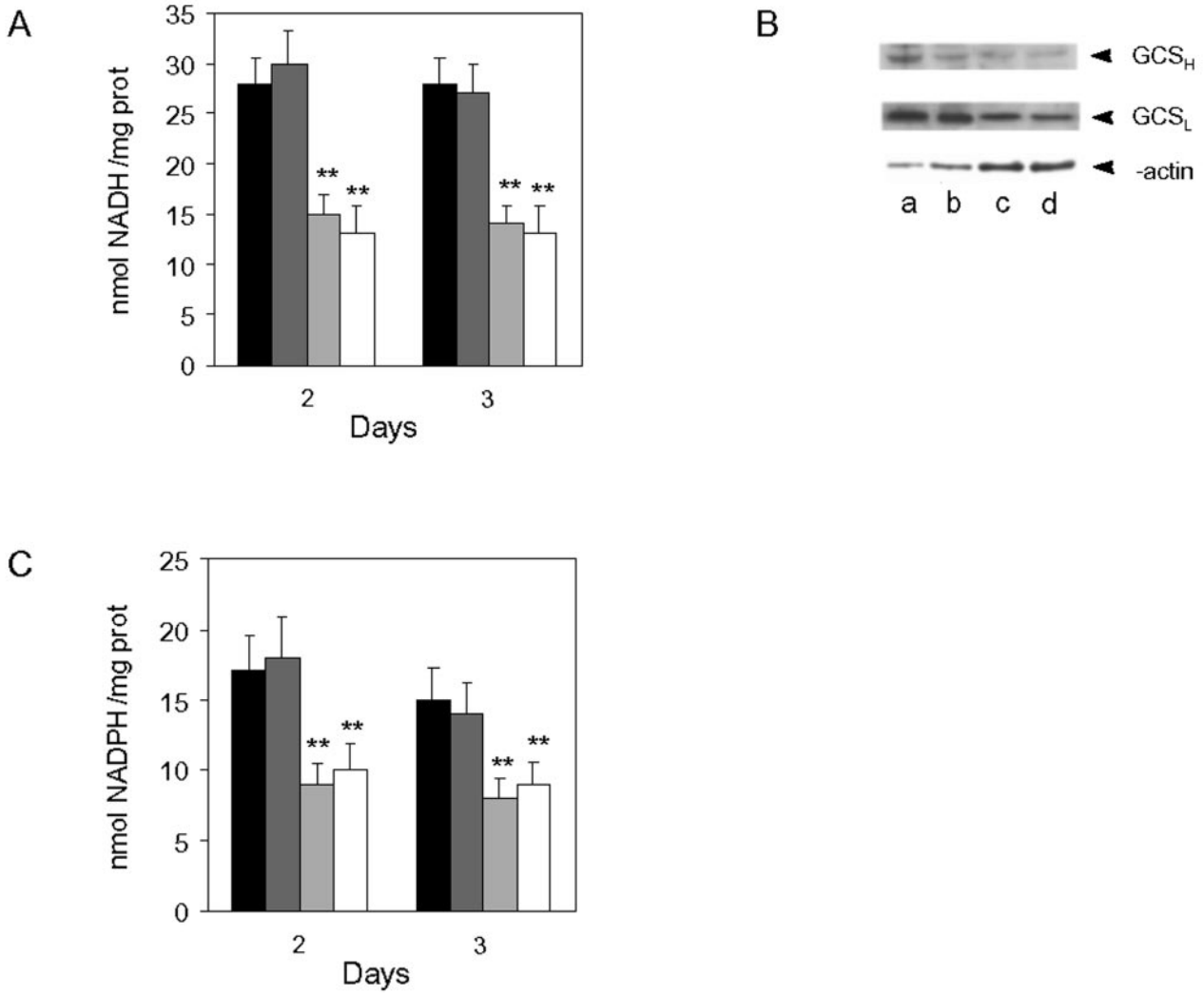

FIG. 3. **Down-regulation of c-Myc reduces the activities of γ-GCS and GSSG reductase.** Enzymatic activity (*A*) and immunoblot assay (*B*) of γ-GCS and enzymatic activity of NADPH-dependent GSSG reductase (*C*) were evaluated in M14 (*black columns*), MN2 (*dark gray columns*), MAS51 (*light gray columns*), and MAS53 (*white columns*) transfectants. On the indicated days, cells were treated for spectrophotometric determinations of enzyme activities as described under "Experimental Procedures" ($n = 6$); **, $p < 0.01$. The blot shown is from a typical experiment of three separate experiments with comparable results.

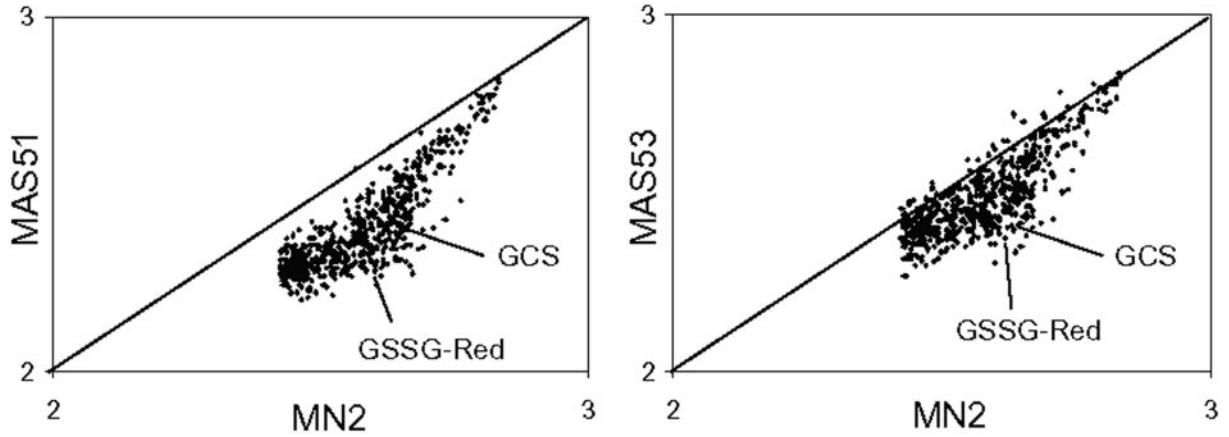

FIG. 4. **Gene expression of γ-GCS (*GCS*) and GSSG reductase (*GSS-Red*) is reduced in clones expressing low amount of c-Myc.** Scatter plots of $\log_{50}$-transformed expression data are shown for MAS51 (*left panel*) and MAS53 (*right panel*) *versus* control transfectant MN2. *Points* placed *below* or *above* the *solid line* represent down- or up-regulated genes in the two clones expressing low c-Myc levels with respect to the control transfectant.

that many genes are down-regulated in the clones expressing low c-Myc with respect to control, including c-*myc* itself and many genes already known as targets of c-Myc. In particular, down-regulation of c-Myc induced a significant reduction of genes of enzymes related to the metabolism of glutathione: γ-GCS, NADPH-dependent GSSG reductase, glutathione

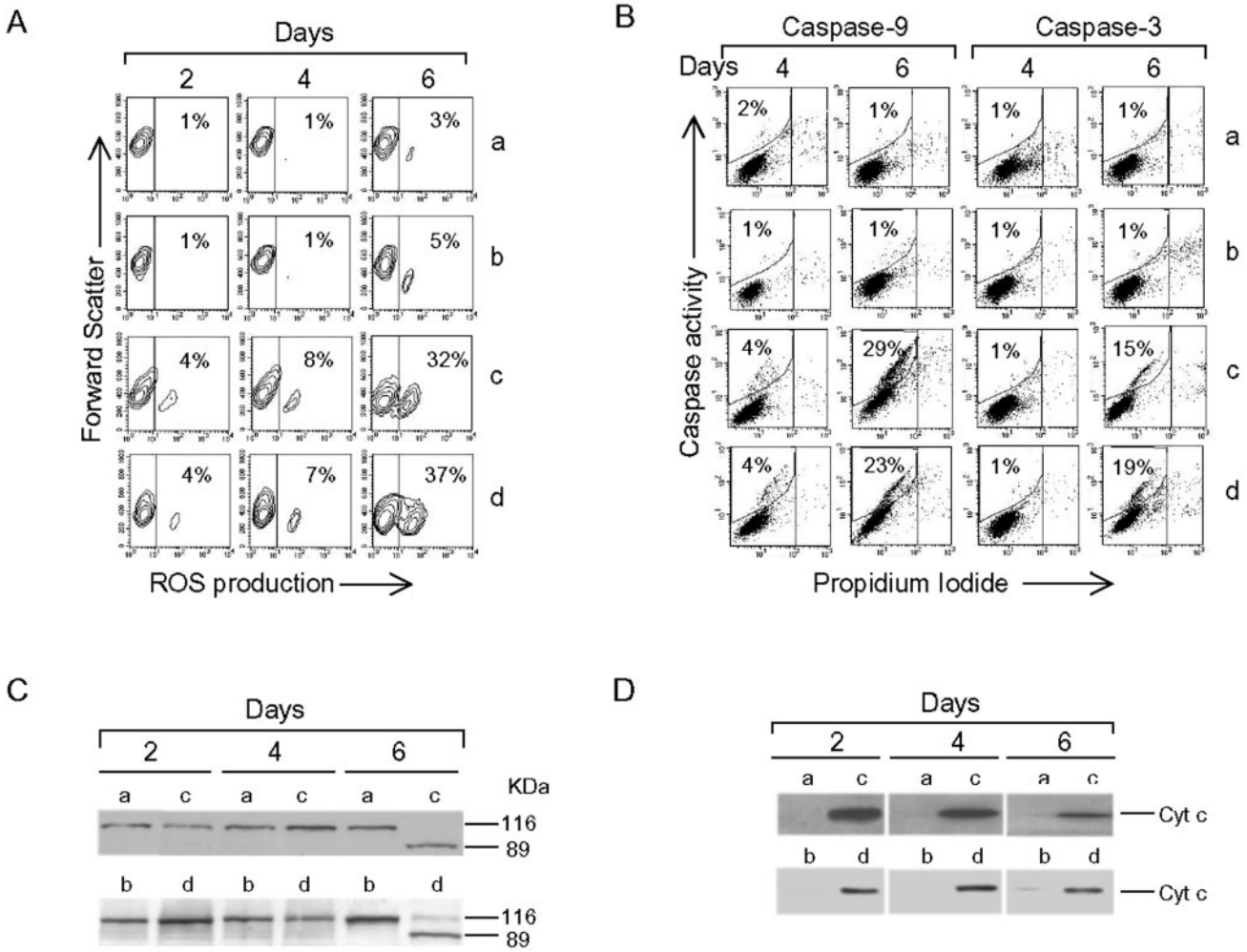

FIG. 5. **Down-regulation of c-Myc triggers apoptosis via the mitochondrial pathway.** The following are shown: flow cytometric analysis of ROS content (*A*) and caspase-9 and caspase-3 activity (*B*); Western blot analysis of PARP cleavage (*C*) and cytosolic cytochrome *c* (*D*) evaluated in M14 (*a*), MN2 (*b*), MAS51 (*c*), and MAS53 (*d*) cells on the indicated days ($n = 5$). Each blot is from a typical experiment of three separate experiments with comparable results.

S-transferase-μ1, -θ1, and -A1, and microsomal glutathione *S*-transferase II.

Down-regulation of c-Myc Commits M14 Melanoma Cells to Apoptosis via the Mitochondrial Pathway—Production of ROS (Fig. 5*A*) progressively increased in the clones expressing low c-Myc after 4 days of growth, reaching significant values (35% more than in control cells) on day 6 of culture. On the contrary, no detectable ROS were produced in the M14 and MN2 control clones during the days of culture. Neither caspase-9 nor caspase-3 was activated until day 4 of growth in all of the cell lines analyzed, regardless of c-Myc expression (Fig. 5*B*). The proteolytic cleavage of the 116-kDa PARP to an 89-kDa product appeared only in MAS51 and MAS53 cells on day 6 of culture, concomitantly with the activation of both caspases (Fig. 5*C*). On the contrary, cytochrome *c* release from mitochondria was detectable as early as day 2 of growth (Fig. 5*D*). The release was concomitant with a decrease in GSH and preceded the surface exposure of phosphatidylserine, as specifically detected by annexin V staining (see Fig. 1*A*).

Activation of Apoptosis in Clones Expressing Low Amount of c-Myc Depends on GSH—When Cys-NAc (5 mM), which is known to support GSH synthesis, or GSH ester (5 mM) was added to transfectants with low c-Myc on day 1 of growth and left in the medium for the following 24 h, the intracellular GSH content was increased to levels as high as those assayed in M14 cells on day 2 of growth (Fig. 6*A*). This effect was maintained for the following days of growth (data not shown) and caused a reduction of apoptosis (Fig. 6*B*). The reversing ability of Cys-NAc and GSH ester was not due to increased expression of c-Myc (Fig. 6*C*). As soon as the GSH concentration rose following both treatments, cytochrome *c* was not efficiently accumulated in the cytosol (Fig. 6*D*). Although both treatments partially reversed the induction of apoptosis in both clones with low c-Myc, the Cys-NAc treatment only was able to completely abolish the intracellular ROS production, whereas the administration of GSH ester reduced it by about 50%. The activities of caspase-9 and caspase-3 were significantly reduced following Cys-NAc or GSH ester treatment, the proteolytic activity being less than 10%.

Transient Down-regulation of c-Myc Induces Apoptosis through a Decrease in GSH—To ascertain whether cell depletion of GSH and induction of the apoptotic program were actually specific events of down-regulation of c-Myc, we generated M14 doxycycline-inducible clones (named MAS IND 1 and MAS IND 18). Both clones, following doxycycline administration, showed a reduction in the protein levels of c-Myc (50–60%) (Fig. 7*A*) and a concomitant decrease in GSH content (Fig. 7*B*). Moreover, a time-dependent increase in the percentage of annexin V+ cells was detected starting from 24 h after c-Myc down-regulation (Fig. 7*B*). Subsequent to cell depletion of glutathione, cytochrome *c* was released from mitochondria, leading to the induction of the downstream events of apoptosis such

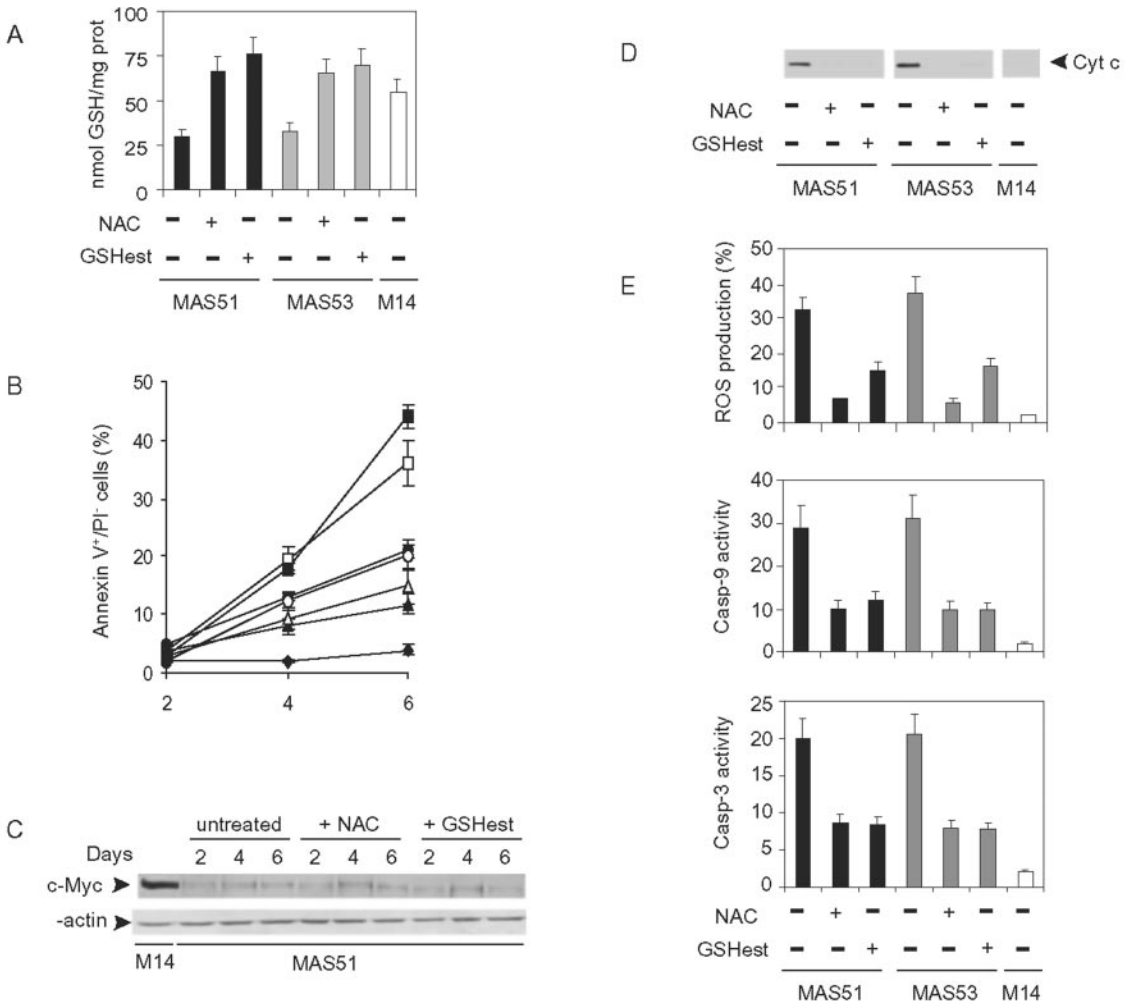

FIG. 6. **Induction of apoptosis depends on cell depletion of GSH.** *A*, intracellular GSH content evaluated in untreated M14, MAS51, and MAS53 or in Cys-NAc-treated (*NAC*) or GSH ester (*GSHest*)-treated cells on day 2 of growth; ($n = 4$). *B*, percentage of apoptosis evaluated by an annexin V/PI assay in M14 (♦), MAS51 and MAS53 untreated (■, □), Cys-NAc-treated (▲, △), and GSH ester-treated (●, ○) transfectants at 2, 4, and 6 days of growth; ($n = 4$). *C*, immunoblot analysis of c-Myc protein expression evaluated in M14 and MAS51 untreated, Cys-NAc-treated, and GSH ester-treated cells on day 2, 4, and 6 of culture. *D*, immunoblot analysis of cytosolic cytochrome *c* performed in M14, MAS51, and MAS53 untreated and Cys-NAc- and GSH ester-treated cells on day 2 of growth. *E*, percentage of ROS production and caspase-9 and -3 activity calculated by flow cytometry in M14, MAS51, and MAS53 untreated and Cys-NAc- and GSH ester-treated cells on day 6 of growth ($n = 4$). Each blot shown is from a typical experiment of three separate experiments with comparable results.

as production of ROS and activation of caspase-9 and -3 (data not shown).

DISCUSSION

The biological effects of c-Myc are clearly due to its ability to affect gene transcription both positively and negatively. Current evidence is in agreement with the notion that deregulation of cell growth and proliferation is critically affected in c-Myc-related tumorigenesis. It is also clear that c-Myc controls or affects other processes that may be highly relevant to its tumorigenic action.

In the present report we demonstrate that down-regulation of c-Myc commits melanoma cells to apoptosis via alteration of the GSH balance in the cell at two key points: GSH biosynthesis and GSH regeneration from GSSG reduction. In fact, by using stable and doxycycline-inducible M14 melanoma cells, we found that the down-regulation of c-Myc induced a decrease in the intracellular GSH content with a concomitant accumulation of its oxidized form. The process is strictly related to down-regulation of c-Myc and not to GSH loss from the dying cells, as clearly evidenced by the kinetics of the appearance of apoptotic cells. We and other authors have demonstrated previously that GSH is efficiently extruded from cells undergoing programmed cell death (26, 27, 29, 35, 36) and that supplementation with GSH precursors or inhibition of GSH efflux leads to inhibition or delay of the death program (30, 37–39).

The observed imbalance in the redox equilibrium of GSH is due to down-regulation of two key enzymes of glutathione metabolism, along with changes observed in other gene transcription, that, as expected, are more related to cell cycle regulators (40). In this context, it should be remembered that another gene involved in redox regulation has recently been reported to be a target of c-Myc, *i.e. PRDX3*, encoding a mitochondrial protein of the peroxiredoxin family (41). As far as γ-GCS and NADPH-dependent GSSG reductase enzyme regulation is concerned, there are no data demonstrating a direct transcriptional regulation of these genes by c-Myc. The results obtained with transient down-regulation of c-Myc (Fig. 7) strongly favor this hypothesis. A rapid decrease of intracellular GSH following deregulation of GSH biosynthesis has recently been demonstrated to be the result of cleavage of the catalytic subunit of γ-GCS by caspase-3 during apoptotic cell death (42). However, under our experimental conditions, the activation of caspase-3 was significant only after 6 days of growth, whereas a decrement in both the protein and activity levels of γ-GCS

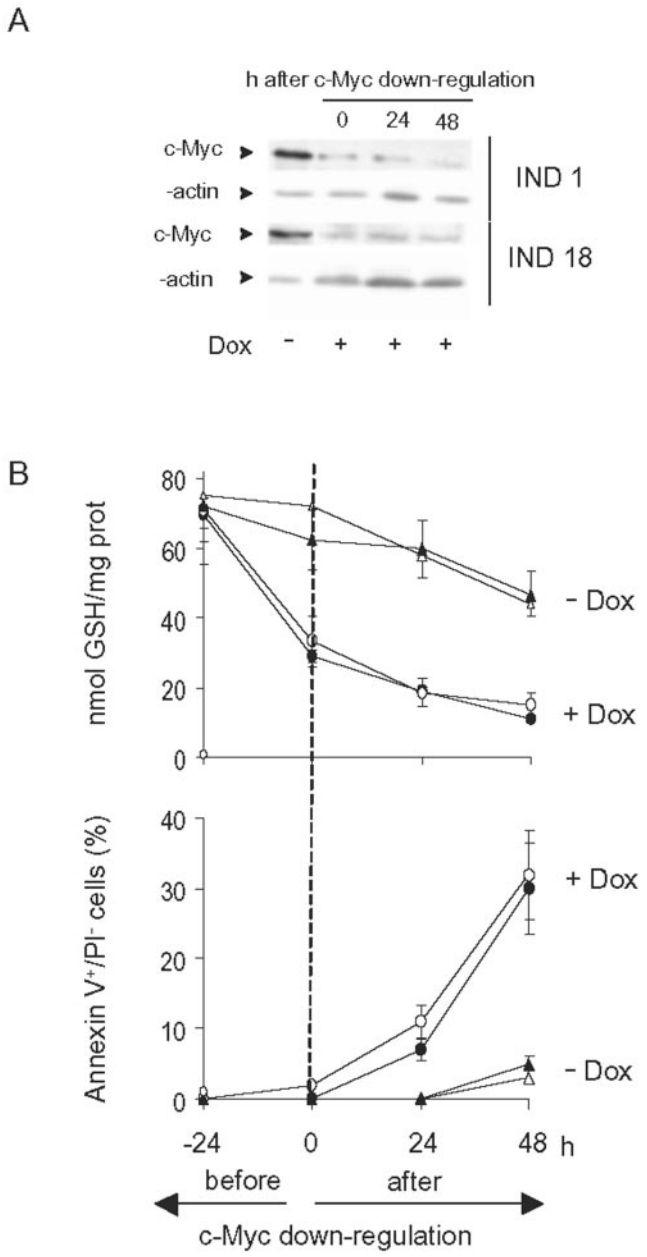

FIG. 7. **Transient down-regulation of c-Myc induces apoptosis through the decrease of GSH.** *A*, Western blot analysis of c-Myc expression levels performed in uninduced and doxycycline-induced (*Dox*) MAS IND1 and MAS IND18 clones. *B*, intracellular GSH content ($n = 4$) and percentage of apoptosis performed by annexin V/PI assay ($n = 4$) evaluated in uninduced MAS IND1 and MAS IND18 (▲, △) cells and doxycycline-induced MAS IND1 and MAS IND18 (●, ○) cells. Upon doxycycline administration, both clones down-regulate c-Myc protein in about 72 h by 50–60% compared with the same uninduced transfectants. This time is conventionally reported as time 0. The blot shown is from a typical experiment of three separate experiments with comparable results.

was detectable as early as 2 days of growth, thus indicating that these decreases are probably a direct consequence of down-regulation of c-Myc. Moreover, the reduction of intracellular GSH content is aggravated by efficient extrusion of GSH, observed as early as 2 days of growth, before the execution of apoptosis. These results are in agreement with previous data demonstrating that cells stimulated to undergo apoptosis get rid of their GSH to allow apoptosis to take place (30). However, we previously found that GSH loss may be necessary but not sufficient for triggering apoptosis, because in lowering the GSH content of the cell by L-buthionine-sulfoximine and diethyl maleate we were not able to induce apoptosis in U937 or HepG2 cells (30). This discrepancy may depend on cell context or on the modality by which GSH is lost; some cell types may adapt slowly to a situation of GSH deprivation by setting up other ways of maintaining a correct redox equilibrium, whereas other types, including melanomas, are particularly sensitive to depletion of GSH (43, 44). In our experimental model, the decrease in GSH is strictly associated to apoptosis, because supplementation with Cys-NAc or with GSH ester inhibited apoptosis.

Cell depletion of GSH caused by down-regulation of c-Myc is linked closely to mitochondrial dysfunction. In fact, we found evidence that release of cytochrome *c* from the mitochondria to the cytosol was concomitant with the decrease of GSH. These results are consistent with our previous data demonstrating that a diminution of intracellular GSH content, obtained either by chemical inhibition of its neosynthesis or by eliciting the extrusion of GSH, causes cytochrome *c* release from mitochondria (31). However, we previously demonstrated that cytochrome *c* release is a cellular response to lack of GSH, which can occur even in the absence of cell commitment to apoptosis; thus, cytochrome *c* release *per se* is not sufficient to trigger apoptosis, but other events have to occur as well (31). In our model, cytochrome *c* release is an early event that is followed by ROS production and proteolytic activation of caspase-9 and -3. The increased ROS production in cells deprived of GSH can be ascribed to an alteration of the redox equilibrium following the impairment of systems able to scavenge or detoxify the various reactive oxygen intermediates generated by normal cell metabolism.

In summary, in this study we have outlined the mechanisms by which down-regulation of c-Myc commits melanoma cells to apoptosis. In particular, we have demonstrated that inhibition of the expression of c-*myc* causes deregulation of the biosynthesis of GSH and of the GSH/GSSG ratio. A decrease in GSH and consequent cytochrome *c* release are early events in the apoptotic process, because they precede the surface exposure of phosphatidylserine. On the contrary, ROS production and activation of caspase-9 and -3, occurring later in the apoptotic process, appear as consequences of redox imbalance. On the basis of these results, γ-GCS and GSSG reductase appear to be possible therapeutic targets that can be modulated specifically to reestablish the apoptotic pathway in cancer cells. Moreover, because elevated levels of GSH have also been reported to play an important role in mediating tumor cell resistance to chemotherapy, the effect of down-regulation of c-Myc on GSH may contribute to increase drug susceptibility.

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