Myc Down-Regulation Sensitizes Melanoma Cells to Radiotherapy by Inhibiting MLH1 and MSH2 Mismatch Repair Proteins

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Abstract

Purpose: Melanoma patients have a very poor prognosis with a response rate of <1% due to advanced diagnosis. This type of tumor is particularly resistant to conventional chemotherapy and radiotherapy, and the surgery remains the principal treatment for patients with localized melanoma. For this reason, there is particular interest in the melanoma biological therapy.

Experimental Design: Using two p53 mutant melanoma models stably expressing an inducible c-myc antisense RNA, we have investigated whether Myc protein down-regulation could render melanoma cells more susceptible to radiotherapy, reestablishing apoptotic p53-independent pathway. In addition to address the role of p53 in the activation of apoptosis, we studied the effect of Myc down-regulation on radiotherapy sensitivity also in a p53 wild-type melanoma cell line.

Results: Myc down-regulation is able per se to induce apoptosis in a fraction of the cell population (~40% at 72 hours) and in combination with γ radiation efficiently enhances the death process. In fact, ~80% of apoptotic cells are evident in Myc down-regulated cells exposed to γ radiation for 72 hours compared with ~13% observed after only γ radiation treatment. Consistent with the enhanced apoptosis is the inhibition of the MLH1 and MSH2 mismatch repair proteins, which, preventing the correction of ionizing radiation mismatches occurring during DNA replication, renders the cells more prone to radiation-induced apoptosis.

Conclusions: Data herein reported show that Myc down-regulation lowers the apoptotic threshold in melanoma cells by inhibiting MLH1 and MSH2 proteins, thus increasing cell sensitivity to γ radiation in a p53-independent fashion. Our results indicate the basis for developing new antitumor therapeutic strategy, improving the management of melanoma patients.

Patients with melanoma have a very poor prognosis with a response rate of <1% due to advanced diagnosis (1, 2). This type of tumor is particularly resistant to both conventional chemotherapy (3) and radiotherapy (4), and surgery remains the main choice of treatment for patients with localized melanoma (5). For this reason, a widespread interest arose about biological therapeutic treatment of melanoma, such as biological therapy, immunotherapy, and molecular therapy (1). More recently, defects in cell death pathways have been found in human melanoma, which seems to be responsible for the resistant phenotype (6).

Moreover, evidences suggested that the overexpression of cell cycle–related proteins could be considered of prognostic value for patients with melanoma. In particular, c-myc oncogene is known to play a central role in controlling the proliferation and differentiation of many normal and neoplastic tissues (7). A recent study also showed that the transfection of high copy numbers of the c-myc gene into a human melanoma cell line is correlated with metastatic potential in severe combined immunodeficient mice and therefore seems to be associated with tumor progression (8). Furthermore, Grover et al. (9–12) and Chana et al. (13) have shown that Myc overexpression is correlated with a worse clinical outcome in patients with malignant melanoma. These prognostic studies indicate that Myc plays a key role in melanoma pathogenesis, thus providing a target for antisense oligodeoxynucleotide treatment. Data reported in literature showed that a down-regulation of the Myc protein by using c-myc antisense oligodeoxynucleotides induces apoptosis in BV173 leukemia cells as well as in HL60 (14, 15). Besides, we have shown previously that Myc down-regulation caused a significant inhibition of the cell proliferation and induced apoptosis in human melanoma experimental models, also enhancing cis-diaminedichloroplatinum (II) antitumor efficacy both in vitro and in nude mice (16, 17).
Therefore, Myc seems to be a valuable target to sensitize human melanoma cells to diverse stress, including radiotherapy, offering opportunities for the development of new therapeutic interventions.

Recently, Mac Partlin et al. (18) have reported the binding of the Myc protein to mismatch repair (MMR) protein MLH1. They found that the deregulation of c-Myc produced an inhibitory effect on MMR activity in normal and tumor cells. MSH2 and MLH1 play a central role in correcting DNA mismatches occurring during the DNA replication, safeguarding genomic integrity. In addition, the inactivation of hMSH2 and hMLH1 MMR genes is the most frequent condition for the loss of MMR found in hereditary nonpolyposis colorectal cancer and sporadic tumors (19). Moreover, they were also reported to be involved in the engagement of apoptosis induced by several cytotoxic anticancer agents (18).

On this basis, the aim of this work was to investigate whether in vitro Myc protein down-regulation could render melanoma cells more susceptible to radiotherapy, thus reestablishing apoptotic pathway. Data herein reported show that Myc down-regulation increases melanoma sensitivity to γ radiation by inhibiting MLH1 and MSH2 proteins and activating a p53-independent apoptotic pathway. Our results seem to actually be able of indicating how to develop a new therapeutic strategy to improve the treatment of patients with melanoma.

**Materials and Methods**

**Cell culture.** M14 (16) and SK-MEL28 (20) p53 mutant and A375M p53 wild-type (21) human melanoma cell lines were employed in this study. The status of p53 gene was analyzed in our laboratory. SK-MEL28 was purchased from the American Type Culture Collection (Milan, Italy), and A375M was a kind gift of Dr. M. Paggi (Regina Elena, Rome, Italy).

M14 cell clones stably expressing an ecdysone-inducible c-Myc antisense mRNA were obtained in a previous article (17). Stable inducible expression of c-Myc antisense mRNA in SK-MEL28 was achieved using the same ecdysone-inducible expression system (Invitrogen, San Diego, CA) and described previously for M14 cell line (17). G418/Zeocyn-resistant SK-MEL28 pINDneo control and pINDc-Myc antisense clones were isolated under ecdysone-free conditions after a selection period of 3 weeks. The different cell clones were scored for ecdysone-inducible inhibition of cell proliferation, and the SK-MEL28 pINDc-myc antisense clone, showing the highest inhibition of cell proliferation on hormone exposure (data not shown), was chosen for all experiments. M14 and SK-MEL28 cell clones were cultured in RPMI 1640, and A375M cells in DMEM, supplemented with 10% FCS (Life Technologies, Scotland, United Kingdom) at 37°C in a 5% CO2/95% air atmosphere.

**Treatments and growth inhibition assay.** To choose the radiation dose, which did not produce toxic effect, pINDNeo cells of both M14 and SK-MEL28 lines were seeded in 60 mm Petri dishes at a density of 1.0 × 105 cells per dish and, after 24 hours of cell culture, were irradiated at room temperature by using 6 MV photons at a dose rate of 4.0 Gy/min. The radiation dose homogeneity was estimated at 3% by the use of existing clinical profiles and depth-dose data for the linear accelerator (Varian Clinac 600c/d). PMMA phantom was used to obtain the required ionizing radiation (IR) dose for fraction, and dosimetric measurement by ionization chamber (Scanditronix FC65G and Kodak X-Omat slides) was done. Different radiation doses (5, 10, and 20 Gy) were tested as single dose. After IR, the dishes were immediately put back in the incubator and cell viability was determined daily from day 2 (24 hours after treatment) to day 5 (96 hours after treatment) of culture. Data were evaluated as percentage of control (i.e., absolute treated cell number/absolute control sample). All the experiments were repeated four times and each experimental sample was seeded in triplicate.

To reduce the level of Myc protein, both M14 and SK-MEL28 pINDc-myc antisense were induced with 20 μmol/L hormone (Ponasterone A, ecdysone analogue) given every 24 hours. Both M14 and SK-MEL28 pINDneo clones did not show any cell growth inhibitory effect after Ponasterone A compared with the parental cell line proliferation. Myc down-regulated cells were exposed to IR and the effect on cell viability was evaluated as described above.

**Propidium iodideotoxicity assay.** The capacity of 5, 10, and 20 Gy radiation to produce cell death was determined by Propidium Iodide (PI) staining exclusion test by flow cytometry. Cytotoxicity was defined as the cellular damage identified by PI staining, which shows the loss of structural integrity of the plasma membrane, a typical event of necrotic cell death. Both M14 and SK-MEL28 cell lines were stained with 100 μL of the supravital PI (4 mmol/L) for 3 minutes. After incubation, cells were washed with 5% bovine serum albumin in PBS and kept on ice until flow cytometry analysis.

**Transient transfection.** A375M cell line was used for transient transfections of the pINDc-myc antisense construct. Cells (3 × 105 in 100 mm dishes) were cotransfected the day after seeding with 4 μg pINDc-myc antisense, 4 μg pCAGGS, and 2 μg pCMV-NEGF-p-spectrin plasmids, mixed in Opti-MEM containing 25 μL Lipofectamine reagent, and incubated for 7 hours according to the manufacturer’s instructions. Afterward, the transfected cells were maintained in fresh growing medium for 24 hours, induced with 20 μmol/L Ponasterone A (induction of c-Myc antisense transcription), and exposed to single IR dose (5 Gy). After 48 hours of treatment, the samples were processed for cell cycle and Western blot analyses as described below.

**Cell cycle analysis.** The cell cycle was studied by using both PI staining and bromodeoxyuridine (BrdUrd; BrdUrd; Sigma Chemical Co., St. Louis, MO) incorporation.

As to PI staining, treated and untreated M14 and SK-MEL28 cell clones or A375M transient transfectants were harvested, washed in cold PBS, fixed in 70% ethanol for at least 1 hour, and, after removing alcoholic fixative, stained with a solution containing 50 μg/mL PI (Sigma Chemical) and 75 KU/mL RNase (Sigma Chemical) in PBS for 30 minutes at room temperature in the dark. Samples were then measured by using a FACScan cytofluorimeter (Becton Dickinson, Sunnyvale, CA).

To BrdUrd incorporation assay, pINDNeo and Ponasterone A–induced pINDc-myc antisense clones from both M14 and SK-MEL28 cell lines were exposed to 5 Gy IR dose for 24, 48, and 72 hours. BrdUrd was added to the medium at the dose of 10 μmol/L during the last 30 minutes before analysis. At indicated times, the cells were harvested, washed once in PBS, fixed in 70% ethanol, and stored at 4°C before the analysis. Then, samples were incubated with 4 N HCl for 20 minutes at room temperature to partially denature DNA. Cells were washed once in borax-borate buffer (pH 9.1) to neutralize the acid pH and then once in PBS. Cells were then permeabilized by incubation with complete medium plus 20% FCS and 0.5% Tween 20 for 10 minutes. The samples were then incubated with mouse monoclonal antibody anti-BrdUrd (Roche Diagnostics, Milan, Italy) in complete medium containing 20% FCS and 0.06% Tween 20 (Calbiochem, San Diego, CA) at room temperature for 1 hour. After washing in PBS, cells were incubated with FITC-conjugated F(ab)’ rabbit anti-mouse IgG (DAKO, Glostrup, Denmark) in PBS for 1 hour. Finally, after washing in PBS, cells were stained with a solution containing 5 μg/mL PI and 75 KU/mL RNase in PBS for at least 3 hours. Twenty thousand events per sample were acquired by using a FACScan cytofluorimeter. The top region of the cytograms represents BrdUrd-positive cells.

The percentages of the cell cycle distribution were estimated on linear PI histograms by using the MODFIT software.

**Apoptosis detection.** The induction of apoptosis was studied by using Annexin V and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assays and by determining caspase-3 activation in flow cytometry. Annexin V assay was used to discriminate between...
necrotic and apoptotic cell death. Phosphatidylinerseine externalization is a characteristic of cells undergoing apoptosis and Annexin V has a strong affinity for phosphatidylserine. The simultaneous staining of cells with FITC-Annexin V and PI allowed the resolution of viable cells (double negative), apoptotic cells (Annexin V positive and PI negative), and necrotic cells (PI positive). Cells were resuspended in 200 μL of 1× binding buffer and incubated for 10 minutes with both FITC-Annexin V (10 μL) and PI (10 μL) at room temperature in the dark.

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay was done as described previously (22). Briefly, cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100 in 0.1% sodium citrate, and washed with PBS. Each sample was incubated in 50 μL reaction mixture (terminal deoxynucleotidyl transferase and fluorescein-dUTP) for 1 hour at 37°C, washed in PBS, and measured by flow cytometry using a FACS can cytometer.

For caspase-3 activation, 8.0 × 10^5 cells per sample were pelleted and resuspended in 1 mL of 2% NP40 in PBS for 5 minutes at room temperature. The samples were then centrifuged and the pellet was washed twice with PBS. Labeling was done by adding to the pellets 20 μL of FITC-conjugated anti-active caspase-3 antibody (BD Pharmingen, San Diego, CA) for 1 hour, washing twice with PBS, and then analysis by flow cytometry.

**Western blot and immunoprecipitation analysis.** The samples were solubilized in lysis buffer [10% glycerol, 25 mmol/L Tris-HCl (pH 7.5), 1% Triton X-100, 5 mmol/L EDTA (pH 8), 1 mmol/L EGTA, 1 mmol/L, 4 mmol/L phenylmethylsulfonyl fluoride, 1% aprotinin, 10 mmol/L sodium orthovanadate, 20 mmol/L sodium pyrophosphate] and treated by sonication for 10 seconds. The protein content in the different samples was quantified by using the Coomassie Protein Assay Reagent kit (Pierce Chemical Co., Rockford, IL). Aliquots (40 μg) of protein were subjected to 10% SDS-PAGE. The resolved proteins were blotted to a nitrocellulose membrane and the blots were incubated with primary antibodies such as anti-Myc (clone 9E10, BD Pharmingen), anti- cyclin B1 (clone GNS11, BD Pharmingen), anti-MSH2 (clone G219-1129, BD Pharmingen), anti-MLH1 (clone G168-728, BD Pharmingen), anti-p53 (clone DO-7, BD Pharmingen), anti-Bcl-2 (clone Bcl-2/100, BD Pharmingen), anti-Bax (BD Pharmingen), and anti-β-actin (clone C-11, Santa Cruz Biotechnology, Santa Cruz, CA). Peroxidase-labeled anti-mouse and anti-rabbit IgG (Amersham Life Sciences, Arlington Heights, IL) were used as secondary antibodies. The immunoblots were processed for enhanced chemiluminescence detection (Amersham Life Sciences). The relative amount of protein present in a given sample was quantified by scanning X-rays films and by estimating the relative arbitrary density units normalized to the equivalent actin content in each sample.

Immunoprecipitation was carried out using 1 mg protein extracts and an anti-human Myc monoclonal antibody (clone 9E10, Santa Cruz Biotechnology). Immunocomplexes were bound to protein A-agarose (Amersham Pharmacia Biotech). The immunoprecipitated proteins were then analyzed on 7.5% SDS-PAGE gels followed by incubation with appropriate primary antibody and then with appropriate secondary antibodies.

**Results**

**Low doses of γ radiation inhibit cell proliferation and activate apoptosis in M14 and SK-MEL28 melanoma cell clones.** To investigate the role of c-myc in the susceptibility of melanoma to IR, we have used two human melanoma cell lines (M14 and SK-MEL28) stably expressing a hormone-inducible c-myc antisense mRNA. First, we chose the radiation dose not toxic for the cells. We exposed M14 and SK-MEL28 pINDneo control cells to 5, 10, and 20 Gy IR and examined the effects produced by treatment on cell growth, cell cycle, and cell death at different times after radiation exposure (24, 48, 72, and 96 hours). At each time, cells were harvested and counted by using the trypsin blue dye exclusion test. IR exposure caused a dose-dependent inhibition of the cell proliferation on both M14 and SK-MEL28 pINDneo cell clones (Fig. 1A). Twenty-four hours after exposure of both cell lines to 20 Gy IR, a cell growth inhibition of ~60% was observed. The inhibitory effect become 90% after 96 hours, whereas growth inhibition of ~25% and 40% were obtained after 24 hours of exposure of both M14 and SK-MEL28 pINDneo cells to the lowest IR doses of 5 and 10 Gy, respectively. These latter effects were partially lost during the following days, with cell growth inhibition values evaluated 96 hours after IR treatment of ~15% and 20%, respectively. These data indicate that the inhibition of cell growth is a persistent effect only when the highest radiation dose (20 Gy) is given. Conversely, treatment of the cells with the lowest doses seems to allow a fraction of the cell population to recover from the radiation-induced DNA damage.

To evaluate whether the IR-induced cell growth inhibition could be related to cell cycle perturbations, PI staining and
fluorescence-activated cell sorting analysis were done on M14 and SK-MEL28 pINDneo clones exposed to the IR doses of 5, 10, and 20 Gy.

The IR induced a dose-dependent accumulation of cells in the G2 phase of the cell cycle compared with untreated cells and evaluated 24 hours after treatment (the G2 phase were 29%, 75%, 95%, and 17%, in 5, 10, and 20 Gy and untreated M14 pINDneo cells, respectively; similar results were obtained in the other line). Forty-eight hours after 20 Gy treatment, the G2 accumulation was still markedly evident in both cell lines (78% and 82%, respectively), suggesting that IR led to irreversible DNA damage and could promote cell killing. Consistent with this hypothesis is the increasing toxicity percentages observed in both pINDneo cell clones evaluated as percentage of PI-stained cells (45% and 50% and 90% and 87% in M14 and SK-MEL28 clones at 72 and 96 hours, respectively). PI exclusion test determines the loss of structural integrity of the cell plasma membrane, which represents one of the key marker distinguishing necrotic from live cells. These data finally show that the 20 Gy IR dose is a lethal dose.

Conversely, cells treated by 5 and 10 Gy progressively decreased the G2 phase percentages to 20% and 17%, respectively, within 72 hours from IR treatment and concomitantly increased the G1 phase (40% and 32%, respectively). Consistent with the cell cycle effect is the moderate cytotoxicity produced by 5 and 10 Gy within the same time interval (72 hours from treatment), the percentages of PI-positive cells being <20% and 30%, respectively. These percentages of toxicity persisted even at 96 hours after treatment. Superimposable results were also obtained for SK-MEL28 pINDneo clone.

We then determined whether the doses of 5 and 10 Gy were able of activating apoptotic cell death. Annexin V assay and fluorescence-activated cell sorting analysis were used to this aim. Only the results obtained in M14 pINDneo cell clone are reported in Fig. 1B; a similar behavior was elicited by the SK-MEL28 clones (data not shown). The dose of 5 Gy produced the highest amount of apoptosis associated with lower percentages of necrosis than those induced by the 10 Gy dose. After 48 hours from 5 Gy treatment, apoptosis was 11% and the number of necrotic cells was still negligible (1%) compared with 4% apoptosis and >15% necrosis produced by the 10 Gy dose. At 72 hours, apoptotic cell percentage increased up to 17% with a still low percentage of necrosis when 5 Gy IR were given. On the contrary, after 10 Gy IR, the number of necrotic cells increased to 30%, with a still low amount of apoptosis. Because the dose of 10 Gy provoked cell killing with a high percentage of necrosis, we chose the dose of 5 Gy, which was able to induce only a negligible amount of necrosis although activating apoptotic cell death.

**Myc down-regulation increases melanoma sensitivity to γ radiation treatment.** Myc protein levels were first determined in pINDneo and induced pINDc- myc antisense cell clones of both cell lines treated or not by 5 Gy IR. Western blot analysis evaluated 48 hours after hormone induction revealed a reduction of ~3-fold of Myc protein levels in pINDc- myc antisense compared with control pINDneo cells in both M14 and SK-MEL28.
and SK-MEL28, whereas a minor reduction of Myc protein was observed when Myc down-regulated cells were exposed to 5 Gy IR (2-fold reduction). No significant reduction in the level of Myc protein was observed in pINDneo clones exposed to 5 Gy (Fig. 2).

To determine whether the down-regulation of Myc protein could increase the susceptibility to the radiation exposure in melanoma cells, we investigated the cell growth inhibition and changes in cell cycle profiles at 24, 48, and 72 hours post-IR. The inhibitory effect on the cell growth is shown in Fig. 3A. Exposure of Myc down-regulated M14 and SK-MEL28 cell clones to 5 Gy γ radiation resulted in a higher cell growth inhibition. In fact, the inhibitory effect at 96 hours was ~95% compared with ~10% and 70% of pNDe-myc antisense treated by 5 Gy or hormone-induced pNDC-myc antisense, respectively, in both M14 and SK-MEL28 clones.

The cell cycle analysis was done by BrdUrd incorporation in both M14 and SK-MEL28 cell clones. Figure 3B shows a representative BrdUrd two-dimensional analysis of the M14 cell clones. A significant difference was observed in BrdUrd incorporation between the two pINDneo control and pINDc-myc antisense clones after radiation exposure. Treatment by 5 Gy radiation of Myc down-regulated cells was able to completely impair DNA synthesis. Indeed, the analysis of cytograms at 72 hours after treatment clearly showed that BrdUrd incorporation has been completely inhibited in M14 pINDc-myc antisense after 5 Gy treatment (3%; Fig. 3B, n) compared with untreated cells (50%; Fig. 3B, c). Instead, 5 Gy radiation treatment in M14 pINDneo clone did not significantly impair DNA synthesis, the BrdUrd-positive cells ranging from 44% to 41% within 72 hours from treatment (Fig. 3B, d).

The down-regulation of Myc protein per se inhibited DNA synthesis already at 24 hours from hormone induction (BrdUrd incorporation was 22%; Fig. 3B, g) compared with control cells (56%; Fig. 3B, a). The BrdUrd incorporation was further inhibited after 72 hours of hormone induction (15%; Fig. 3B, i). A similar behavior was elicited by SK-MEL28 cell clones treated by 5 Gy IR (data not shown). The inhibition of BrdUrd incorporation observed in Myc down-regulated cells after 5 Gy radiation is consistent with a reduced cell percentage in S phase observed in both cell lines as estimated by applying to each PI histogram the MODFIT software (Fig. 3C). This analysis also showed the characteristic G2 block produced by IR treatment on both cell lines (~30% and 35%, respectively), which was completely overcome at 72 hours after treatment in both cell lines. As shown in Fig. 3, the G2 accumulation increases when γ radiation was given to Myc down-regulated cells. This effect was particularly evident at 24 hours post-IR (57% and 55%, respectively). At 72 hours post-IR, both M14 and SK-MEL28 Myc down-regulated cells showed a significant depletion of the cells from the G2 phase (25% and 28%, respectively) accompanied by an increased percentage of sub-G1 cells (data not shown), indicating that Myc down-regulated cells are not able to recover from the radiation-induced G2 block. Besides, in combination with IR treatment, Myc down-regulation also produced an accumulation of cells in the G1 phase (>60%) with a depletion from the S phase (~6% in both lines), thus demonstrating that the inhibition of Myc protein prevented the G1-S transition.

To better investigate the molecular mechanisms underlying the G2-phase accumulation after IR treatment in Myc down-regulated cells, we studied the expression of cyclin B1 in pINDneo and hormone-induced pINDc-myc antisense clones exposed or not to 5 Gy IR. To do this, we chose the M14 cell line because the response of both M14 and SK-MEL28 cell lines to IR treatment was superimposable. Western blot analysis of cyclin B1 was done at 12 and 24 hours after hormone induction and/or radiation treatment (Fig. 4). Cyclin B1 levels significantly increased after IR exposure compared with pINDneo control cells. This effect was particularly evident at 24 hours from treatment, indicating that a stabilization of cyclin B1 due to the block in the G2-M progression has been caused by γ radiation treatment. When 5 Gy IR were given to Myc down-regulated cells, a similar cyclin B1 stabilization (3-fold increase) was achieved already at 12 hours post-IR. On the contrary, this effect slightly decreased at 24 hours, although a marked G2 accumulation (57.5%) was still evident. It is likely that a cellular input to undergo apoptosis have been generated during G2 phase. In fact, it has been reported recently as a proapoptotic role for cyclin B1 (25).

To better investigate the activation of apoptosis in Myc down-regulated cells treated with γ radiation, terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay was done. We have shown previously that Myc down-regulation obtained by treatment with c-myc antisense oligodeoxynucleotides or via a hormone-inducible system is able to activate the apoptotic program by inhibiting the progression of cells through the cell cycle (16, 17). Figure 5 shows the percentage of apoptotic cells in pINDneo control and in pINDc-myc antisense in both M14 and SK-MEL28 cell lines treated or not with 5 Gy γ radiation and evaluated at 24, 48, and 72 hours from treatment. The γ radiation treatment activates the apoptotic program, but it is evident that only a slight percentage of cells underwent apoptosis, being cell death <13% until 72 hours in both M14 and SK-MEL28 cell lines. On the contrary, Myc down-regulation is able per se to induce apoptosis in a fraction of the cells (~40% at 72 hours) and in concert with γ radiation enhances the death process efficiently. In fact, ~80% of apoptotic cells are evident at 72 hours in both cell lines.

**Myc down-regulation favors ionizing radiation–induced apoptosis by affecting mismatch repair proteins.** To address whether Myc down-regulation activates apoptosis in melanoma cells by
interfering with the repair of IR-induced DNA damage, we have studied MSH2 and MLH1 MMR protein levels after radiation treatment in control and Myc down-regulated M14 cells. Recent data from Mac Partlin et al. (18) showed that Myc interacts with the DNA MMR protein MLH1 by inhibiting its activity in normal and tumoral cells. Western blotting of both MSH2 and MLH1 proteins normalized versus \( h \)-actin levels revealed that the amount of both proteins increased after radiation treatment (2-fold at 48 hours post-IR) compared with untreated cells, indicating that a repair process was activated by γ radiation. Conversely, Myc down-regulation produced a significant reduction of both MSH2 and MLH1 protein levels (>8-fold for combined treatment). In response to IR, Myc down-regulated cells reduced both MSH2 and MLH1 proteins as well at levels comparable with that of the Myc down-regulated cells (Fig. 6A). This effect was already evident at 6 hours post-IR and was maintained up to 48 hours, suggesting a mechanism of regulation of these proteins by Myc.

Hypothesizing a model in which Myc could interact with MLH1 and/or MSH2, we immunoprecipitated Myc protein and studied the formation of eventual immunocomplexes between Myc and MSH2 and/or MLH1 proteins. Indeed, we found that Myc was able to bind MLH1 MMR protein as shown by the analysis of Myc immunocomplexes (Fig. 6B), whereas no evidence of interaction was found between Myc and MSH2, thus suggesting a major role of MLH1 protein in the repair of IR-induced DNA damage in melanoma cells.

To elucidate whether the decrease of MSH2 and MLH1 levels following Myc down-regulation could be ascribed to an inhibition of their transcription, we did a semiquantitative reverse transcription-PCR analysis of total RNA extracted from pINDneo control and hormone-induced pINDc-myc antisense M14 clones. The PCR amplification was done at various numbers of cycles as indicated. The PCR-amplified MLH1 cDNA was detectable in pINDneo control cells already at 28 cycles of amplification, whereas no detectable amounts of PCR-amplified MLH1 cDNA were obtained after the same number of amplification cycles in Myc down-regulated cells. A barely detectable PCR-amplified MLH1 cDNA was observed in Myc down-regulated cells at 32 cycles, demonstrating that MLH1 transcription is regulated by Myc. Conversely, consistent with
the lack of interaction between Myc and MSH2, we found that transcription of MSH2 MMR protein was not significantly affected by Myc modulation (Fig. 6C). Cyclophilin was used as an internal control for the amount of RNA used in all reverse transcription-PCR reactions.

**Myc down-regulation activates apoptotic program via mitochondrial pathway.** To elucidate the mechanisms by which Myc down-regulation cooperates with γ radiation to induce apoptosis, we investigated the mitochondrial apoptotic pathway. To this aim, caspase-3 activation and Bcl-2 and Bax protein expression were analyzed in M14 pINDneo and hormone-induced pINDc-myc antisense treated or not by 5 Gy IR.

Western blot analysis of Bcl-2 and Bax levels are shown in Fig. 7A. The analysis was done also at early times after treatment (6 and 12 hours). A sustained and gradual decrease of Bcl-2 level was observed in Myc down-regulated cells after 5 Gy radiation, evident already at 12 hours after treatment and maintained until 48 hours. The reduction of Bcl-2 expression was ~10-fold at 48 hours, whereas a slight increase in the protein level was detected after 6 hours of treatment, suggesting that the apoptotic process could be activated by the cell in response to DNA damage only secondarily. No appreciable changes in Bax protein expression in all samples at all times of analysis were observed.

Flow cytometry analysis of active caspase-3 was done at 24, 48, and 72 hours after IR exposure. As shown in Fig. 7B, no evidence of active caspase-3-positive cells was detected in pINDneo after 24, 48, and 72 hours of IR treatment (percentages of positivity were 3%, 4%, and 3%, respectively).
compared with untreated cells (percentages of positivity were 2%, 3%, and 2%, respectively). On the contrary, the percentage of active caspase-3-positive cells increased to 50% and 64% in pINDc-myc antisense at 48 and 72 hours after hormone induction, respectively. Only a slight increase of the active caspase-3-positive cells was observed when Myc down-regulated cells were exposed to 5 Gy radiation, being 64% and 78% at 48 and 72 hours after treatment, respectively, thus suggesting that the Myc down-regulation per se activates a mitochondrial pathway of apoptosis.

Apoptosis induced by Myc down-regulation in melanoma cells is independent from p53. Because it has been proposed that p53 and MSH2 could be linked in a regulatory feedback loop, showing a dual role of p53 in the regulation of growth and DNA repair processes (26), we examined the p53 protein levels in the M14 pINDneo and hormone-induced pINDc-myc antisense clones treated or not with 5 Gy IR (Fig. 6 A). As expected, no modulation of p53 protein levels was observed in all of the analyzed samples, indicating that the endogenous p53 protein is mutant and consequently not functionally active. In addition, the levels of expression of the protein were significantly high, suggesting a stabilization of the protein in this model. Indeed, the mutant genomic sequence of p53 gene has been shown by direct sequencing of PCR-amplified products. Consistent with data obtained by Western blotting analysis, the sequencing of exons 5 to 8 detected a point mutation at codon 266 of exon 8 (GGA-to-GAA; Gly-to-Glu), thus confirming the inactivation of p53 protein (data not shown).

To directly address the role of p53 in the activation of apoptosis induced by down-regulating Myc, we sought to determine the effect of Myc inhibition in the p53 wild-type A375M melanoma cell line. The pINDc-myc antisense and pVgRXR expression vectors were transiently cotransfected with the green fluorescent protein (GFP)-spectrin expression plasmid in A375M cells and the DNA content profile of transfected cells was analyzed by two-color flow cytometry (Fig. 8A). Gating out GFP-positive cells and analyzing the cell cycle distribution of this population 48 hours after exposure of hormone-induced A375M pINDc-myc antisense cells to 5 Gy IR, we showed an enhancement of the apoptosis (40%; Fig. 8A, l) compared with the A375M Myc down-regulated cells (24%; Fig. 8A, i). By contrast, undetectable apoptosis was observed in A375M GFP-positive cells transfected with the empty vector after IR treatment (8%; Fig. 8A, h) as well as in the untreated pINDneo-transfected A375M cells. The cell cycle phase percentages estimated on the DNA profiles of GFP-positive cells as referred to Fig. 8A are summarized in Fig. 8B.

Western blot analysis of both MSH2 and MLH1 proteins in A375M pINDneo-transfected cells treated with 5 Gy IR confirmed the increase of the expression levels of both proteins of ~2-fold as normalized versus β-actin levels, whereas significant reductions (>8-fold) of both MSH2 and MLH1 protein levels occurred in A375M Myc down-regulated
cells alone or in combination with 5 Gy radiation. A significant reduction of Bcl-2 expression was also observed in A375M Myc down-regulated cells treated or not with 5 Gy IR. Increased expression levels of wild-type p53 was shown only in 5 Gy IR–treated A375M pINDneo-transfected cells, indicating that the p53 pathway is functional in this cell line (Fig. 8C).

Taken together, these data suggest that Myc down-regulation sensitizes melanoma cells to the radiation treatment by inhibiting MLH1 and MSH2 proteins, which, as they not correct IR mismatches, render these cells more prone to radiation-induced apoptosis.

Discussion

Melanoma is a malignant tumor type characterized by a poor prognosis partly due to ineffective radiotherapy and chemotherapy (3, 4), although radiotherapy is widely applied for treatment of melanoma patients. The effectiveness of radiotherapy depends on many factors, such as cellular levels of O2 and pH (27–29). Recently, it has been reported that several molecular factors, such as the cell cycle regulators, may also affect the radiosensitivity of cancer cells. In this context, c-myc oncogene, known to play a central role in controlling proliferation, differentiation, and apoptosis, seems to be one possible candidate. In particular, the modulation of Myc oncoprotein has been shown to sensitize human melanoma cells to diverse stress, including chemotherapy agents (30, 31).

In our work, we have studied the effect of Myc down-regulation in combination with radiotherapy on human malignant melanoma cells. To establish whether Myc down-regulation is capable to sensitize cancer cells to radiation treatments, we have used two stable inducible expressing c-myc antisense mRNA melanoma cell clones derived from the p53 mutant M14 and SK-MEL28 cell lines characterized by a moderate radiosensitivity (32). In this article, we show that decreased level of Myc protein renders melanoma cells more susceptible to radiotherapy, inhibiting MLH1 and MSH2 MMR protein levels and reestablishing apoptotic pathways.

To determine that the nontoxic radiation dose is able to induce apoptosis, we tested the doses of 5, 10, and 20 Gy. We found that the dose of 5 Gy is the less toxic one and is able to moderately inhibit cell growth (~20% and 25% growth inhibition in the two cell lines) and activate apoptosis at an extent of ~20% in both cell clones. On the other hand, the dose of 20 Gy, which caused the highest and persistent inhibition on cell growth (>70%), seemed to be highly toxic as shown by PI staining (~90% at 96 hours).

![Fig. 8. A, effect of ectopic inducible c-myc antisense mRNA expression in the p53 wild-type A375M cell line. These cells were cotransfected with pIND-c-myc antisense, pMV RXR, and pCMVEGFP-spectrin plasmids; after transfection, where needed, cells were exposed to hormone induction and to 5 Gy IR. Analysis was conducted 48 hours following transient transfection and treatments. Untransfected cells (a and f) are used as negative control for setting the gating region. GFP-positive cells (top) were analyzed for DNA content distribution by flow cytometry. Top, representative GFP-spectrin fluorescence cytograms; bottom, corespective DNA histograms of the GFP-positive cells. (b and g) pINDneo, (c and h) pINDneo plus 5 Gy, (d and i) induced pIND-c-myc antisense, and (e and l) pIND-c-myc antisense plus 5 Gy. B, cell cycle distribution estimated on the DNA histograms as in A by using the MODFIT software. C, Western blot analysis of the indicated proteins extracted from samples treated as in A. Proteins (70 μg) were loaded in each lane and β-actin was used as control for loading equal amounts of proteins. The experiment was repeated thrice showing similar results. Band intensities were measured by densitometry.](www.aacrjournals.org/clin cancercancerres.aacrjournals.org Downloaded from clin cancerres.aacrjournals.org on April 14, 2017. © 2005 American Association for Cancer Research.)

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after treatment). Our data agree with Louagie et al. (33) who showed apoptosis when lymphocytes were given the dose of 5 Gy or necrosis when the lymphocytes were treated by 20 Gy IR. There are different molecular events that occur following IR, which, affecting cell cycle, can lead to DNA damage repair or death. If the DNA damage is too massive and cannot be repaired, cell cycle checkpoints are activated to induce permanent cell cycle arrest, which is followed by death in the attempt to eliminate such severely damaged cells (34, 35).

The combination of Myc down-regulation and radiotherapy produced a greater inhibitory effect on cell proliferation of melanoma cells than the two treatments given separately, indicating a synergism of the two agents used in combination. Myc levels in our models were unaffected by the only IR treatment, even if Calaf et al. reported an increase of Myc protein expression after radiation treatment in MCF-10F breast cancer cell (36). Confirming previously published results, hormone-induced cell clones showed a significant reduction of Myc protein expression. Interestingly, the reduction of Myc protein in hormone-induced pLNDC-myc antisense clones treated by 5 Gy IR was slighter than in the only Myc down-regulated clones, suggesting a role of Myc in the G2 cell cycle phase. Our data clearly show that tumor cells exposed to the lowest radiation dose partially recovered the IR-induced G2 arrest, whereas the Myc protein reduction abrogated the ability of the cells to overcome the G2 block. This G2 block seems to be irreversible, because at 72 hours from the start of treatment a fraction of the cells, not able to repopulate the cell cycle, died from apoptosis.

Recently, it has been reported that the cellular decision to activate apoptosis or proceed into mitosis is due to cyclin B1 in several mouse and human hematopoietic cells as well as in primary thymocytes (25). According to other articles, we found an accumulation of cyclin B1 following IR exposure, particularly evident at 24 hours after treatment, whereas Myc down-regulated cells treated by 5 Gy display an earlier (12 hours after treatment) increase in cyclin B1 levels, which successively decreased after 24 hours. It is likely that these cells progressively escaped from G2 block, undergoing apoptotic cell death to eliminate the damaged cells. This hypothesis is supported by the dramatic increase of the proportion of apoptotic cells during the combined treatment, associated with a marked loss of cells with S and G2 DNA content at 72 hours. Although these data are in conflict with other articles, which showed that c-myc is necessary for the activation of DNA damage-induced apoptosis in G2 phase (37–41), other studies on an immortal 32D murine myeloid cell line have shown that Myc expression itself had no effects on cell cycle arrest following IR treatment (42). On the other hand, previous articles showed both in vitro and in vivo that antisense phosphorothioate oligodeoxynucleotides targeted to the c-myc mRNA, inhibiting cell proliferation, render melanoma cells more sensitive to cis-platinum treatment (43, 16).

The enhanced apoptotic effect elicited by the combination is to be ascribed to the different mechanism of action of the two treatments. In fact, cell cycle analysis after IR treatment revealed the characteristic G2 phase block after 24 hours, although this block was in part circumvented as shown by the cell cycle repopulation during the days following the treatment. Instead, the down-regulation of Myc, inhibiting cell cycle progression, as shown by the accumulation in G1 phase, prevented the MLH1- and MSH2-mediated IR damage repair. In addition, we show that MLH1 transcription is regulated by Myc as shown by the decreased MLH1 cDNA amplified after Myc down-regulation. It is also reported in literature that the promoters of MMR genes show potential binding sites for transcriptional activators (44–46), thus suggesting MLH1 as a Myc target gene. In addition, Mac Partlin et al. recently reported that Myc is able to bind MLH1 MMR protein, thus inhibiting its activity (18). Our findings are in accord with those of Mac Partlin et al. because we also found that Myc can form complexes with MLH1 yet would be unable to bind MSH2. However, we found that both MLH1 and MSH2 proteins were reduced. It is likely that reduction of MSH2 protein could be successive to that of MLH1 and the inability of MLH1 to bind DNA in the damage repairing processes could determine the MSH2 reduction, thus preventing the stabilization of DNA filaments needed to the occurring DNA repair events. Recent studies suggest that the maintenance of an appropriate balance of the components of the human MMR system is a critical step for its proper repair functions (47–49). The consequence of the MLH1 and MSH2 protein inhibition is the prevention of IR mismatches correction occurring during DNA replication, thus rendering the cells more prone to radiation-induced apoptosis. In fact, because the MMR repair system ensures replication fidelity by correcting postreplication errors that have escaped the DNA proofreading function of DNA polymerase, loss of MMR protein induced by Myc down-regulation could lead to accumulate DNA damage and does not allow damage repair, thus inducing apoptosis. Our data are also in agreement with Hawn et al. who reported recently that the MMR system interacts with the G2 checkpoint providing an opportunity of DNA repair in G2 (50).

The apoptosis induced by Myc down-regulation occurs via a mitochondrial pathway as shown by the activation of caspase-3, a key event in the death process. Indeed, in our model, we observed a significant percentage of positivity of active caspase-3 protease in Myc down-regulated cells compared with control cells. The IR exposure alone is not able to induce the caspase-3 activation, whereas the caspase-3 activation occurs in Myc down-regulated cells, which are unable to recover IR damage. The involvement of the mitochondrial pathway in the IR-induced apoptosis in Myc down-regulated cells is also shown by the decrease of the antiapoptotic Bcl-2 protein. Firstly, Myc down-regulation impairs MLH1 and MSH2 proteins, and only secondarily, the apoptotic processes are activated by suppressing the expression of the apoptotic antagonist Bcl-2.

This apoptotic effect occurs independently by the p53 pathway. p53 is another gene critical for the DNA repair processes. One central function of the p53 protein is to bind DNA and regulate genes that control cell cycle and cell death. p53 has been defined as the guardian of the genome, because it can induce cell cycle arrest at G1 phase and allow cells to repair DNA damage induced by IR (51, 52). However, point mutations and/or p53 gene deletions occur in ~50% of solid tumors, including melanoma (53–56). Recently, Zhu et al. have reported a high incidence of abnormal expression of MSH2 protein in leukemia cells with p53 mutant (57).
data clearly show that the modulation of MSH2 and MLH1 proteins was p53 independent and that Myc down-regulation induces apoptosis in a way that does not require the functionality of p53 in the melanoma cells. Further understanding of the molecular mechanism underlying the sensitization of tumor cells to apoptosis could represent the basis for developing new antitumor therapeutic strategy aimed to reestablish apoptotic pathways usually altered in cancer cells.

Acknowledgments

We thank Daniele Khouri for technical assistance and Dr. Cecilia Verga Falzaccapa for electronic support.

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Cancer Therapy: Preclinical

Clin Cancer Res 2005;11(7) April 1, 2005 2766
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Myc Down-Regulation Sensitizes Melanoma Cells to Radiotherapy by Inhibiting MLH1 and MSH2 Mismatch Repair Proteins

Barbara Bucci, Igea D'Agnano, Donatella Amendola, et al.


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