Decitabine, differently from DNMT1 silencing, exerts its antiproliferative activity through p21 upregulation in malignant pleural mesothelioma (MPM) cells


**A R T I C L E   I N F O**

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**Abstract**

Malignant pleural mesothelioma (MPM) is a locally aggressive neoplasm, principally linked to asbestos fibres exposure. Strong evidences associate this pollutant with induction of DNA breaks, aberrant chromosomes segregation and important chromosomal rearrangements, considered crucial events in malignant transformation. A considerable contribution to cellular transformation in MPM is also given by the presence of high genomic instability, as well as by the increased DNA methylation, and consequent decreased expression, of tumor-suppressor genes. In this study we first demonstrated that MPM cells are characterized by a decreased methylation level of pericentromeric DNA sequences which can justify, at least in part, the genomic instability observed in this neoplasia. Concomitantly, we found a paradoxical increased expression of DNMT1, the most expressed DNA methyltransferases in MPM cells, DNMT3a and all five isoforms of DNMT3b. Thus, we compared two experimental strategies, DNMT1 silencing and usage of a demethylating agent (5-aza-2′-deoxycytidine or Decitabine), both theoretically able to revert the locally hypermethylated phenotype and considered potential future therapeutic approaches for MPM. Interestingly, both strategies substantially decrease cell survival of MPM cells but the antitumor activity of Decitabine, differently from DNMT1 silencing, is mediated, at least in part, by a p53-independent p21 upregulation, and is characterized by the arrest of MPM cells at the G2/M phase of the cell cycle. These results indicate that the two approaches act probably through different mechanisms and, thus, that DNMT1 silencing can be considered an effective alternative to Decitabine for cancer treatment.

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**1. Introduction**

Malignant pleural mesothelioma (MPM) is an aggressive neoplasia originating from pleural mesothelial cells and epidemiologically associated with asbestos fibres exposure. The molecular mechanisms responsible for cellular transformation are poorly understood but there are evidences that strongly link the asbestos fibres with induction of typical chromosomal aberrations found in MPM cells [1,2].

In the last years, many evidences have been accumulated about the epigenetic alterations of cancer cells. Regarding the genomic DNA methylation, tumor cells are paradoxically characterized by two distinct phenomena: hypermethylation of CpG islands, often in the contest of tumor-suppressor gene promoters, and hypomethylation of CpG dinucleotides that are highly represented in pericentromeric regions, such as satellite 2 repeats [3–6].

Functionally, these perturbations may be involved, respectively, in tumor-suppressor genes silencing and genomic destabilization, crucial events in tumor development. Mesothelioma cells seem to recapitulate the epigenetic features of solid tumors since the presence of high genomic instability and a pattern of transcriptionally repressed tumor-suppressor genes, through DNA hypermethylation, has been demonstrated [7–12].

The molecular mechanisms responsible for the altered DNA methylation pattern in cancer are still unknown. Genetic alterations of DNA methyltransferases (DNMTs) coding sequences seem to be not involved in this phenomenon: human Immunodeficiency, Centromeric instability and Facial abnormalities (ICF) syndrome remains the unique disease in which satellite 2 demethylation and genomic instability are correlated to DNMT3b mutations, even if in only 60–70% of ICF patients [13–15].

Moreover, altered pattern of DNA methyltransferases expression was reported as possible mechanism responsible for either decreased global DNA methylation (e.g. DNMT3b4 dominant-negative isoform overexpression in hepatocarcinoma, DNMT3a loss of expression in glioblastoma) and promoter-associated CpG island
hypermethylation (e.g. overexpression of DNMT1 in almost all kind of neoplasia) [4,16].

Mainly, two strategies have been considered to revert the local hypermethylation in cancer: usage of demethylating agents, such as 5-aza-2′-deoxycytidine (or Decitabine), and interference with DNMTs expression. Decitabine has been tested in several Phase I, II and III clinical trials founding the most promising benefits in leukemia patients, specially those affected by myelodysplastic syndrome (MDS) [17–19]. For this reason Decitabine has been approved for the treatment of MDS showing excellent capability to reactivate the expression of several methylated genes [20]. Although the therapeutic abilities of Decitabine for the treatment of hematopoietic malignancies have been demonstrated, the activity in solid tumors remains unclear and severe toxicities related to prolonged myelosuppression have to be considered [21]. Recently, the potential therapeutic properties of Decitabine have been investigated in a Phase I trial study involving patients with thoracic malignancies, MDS cases included [22]. With a similar aim, DNMT1 silencing procedure has been transiently applied to a variety of cellular models (e.g. lung cancer, breast cancer, gastric cancer) obtaining interesting results in terms of decreased proliferation and induction of programmed cell death [23–25]. In this study we addressed the question whether Decitabine treatment or DNMT1 silencing could be considered equivalent strategies for cancer treatment. We first characterized the epigenetic status of MPM cells monitoring both satellite 2 methylation levels, as marker of genomic stability, and DNMTs expression pattern. Secondly, we compared the biological MPM cellular response to either DNMT1 silencing or demethylating (Decitabine) treatment. Finally, we investigated the role of p21 expression in the MPM antiproliferative response to Decitabine.

2. Materials and methods

2.1. Cell cultures and treatments

Immortalized human malignant pleural mesothelioma NCI-H28 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Primary normal mesothelial cells (HMC2) and primary MPM cells (MES-SS 01, MES-PR 99) were established from pleural effusions and tumor specimens, respectively, and characterized as previously described [26]. All cellular populations were cultured in DMEM (Cambrex, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (FBS), 1% penicillin–streptomycin, 1% glutamine. Decitabine (or 5-aza-2′-deoxycytidine–Sigma, Saint Louis, MO, USA) treatments were performed at indicated doses and timing, adding the compound (Fig. 1b). However, DNMT1 resulted the most expressed form of DNA methyltransferase (>85–90% of total DNMTs) representing the main source of methyltransferase activity in MPM cells, compared with normal mesothelial cells (HMC2) (Fig. 1a).

In order to establish differences in the expression of DNA methyltransferases between normal and tumor cells, that could justify the observed loss of satellite 2 methylation, we analyzed the mRNA levels of DNMT1, DNMT3a and all five isoforms of DNMT3b, by real-time quantitative RT-PCR, in either primary MPM cells (MES-SS 01, MES-PR 99) and in H28 immortalized MPM cell line, if compared with normal mesothelial cells (HMC2) [30,31]. The methylation status of satellite 2 sequences was investigated by a methylation-sensitive endonuclease-based assay revealing decreased CpG methylation levels in two primary malignant mesothelioma cellular populations (MES-SS 01, MES-PR 99) and in H28 immortalized MPM cell line, if compared with normal mesothelial cells (HMC2) (Fig. 1a). In order to establish differences in the expression of DNA methyltransferases between normal and tumor cells, that could justify the observed loss of satellite 2 methylation, we analyzed the mRNA levels of DNMT1, DNMT3a and all five isoforms of DNMT3b, by real-time quantitative RT-PCR, in either primary MPM cells (MES-SS 01, MES-PR 99) and normal mesothelial cells (HMC2). Primary MPM cells showed an increased expression of DNMT1 (4.4–7.2 folds) concomitantly with a less pronounced increased expression of DNMT3a (1.7–3.8 folds) and variable higher levels of DNMT3b (2–6.8 folds) (Fig. 1b). However, DNMT1 resulted the most expressed form of DNA methyltransferase (>85–90% of total DNMTs) representing the main source of methyltransferase activity in MPM cells (Fig. 1b).

3. Results

3.1. Satellite 2 pericentromeric DNA hypomethylation and DNMTs altered expression in MPM cells

Repetitive (ATTCCATTCG)$_2$ DNA sequence is highly represented in regions localized in the pericentromere of chromosome 1 and 16, named satellite 2 [30,31]. The methylation status of satellite 2 sequences was investigated by a methylation-sensitive endonuclease-based assay revealing decreased CpG methylation levels in two primary malignant mesothelioma cellular populations (MES-SS 01, MES-PR 99) and in H28 immortalized MPM cell line, if compared with normal mesothelial cells (HMC2) (Fig. 1a).

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3.2. Stable silencing of DNMT1 expression induces decreased cell survival and programmed cell death of MPM cells

To evaluate the importance of the described DNMT1 accumulation in MPM cells, we stably silenced the expression of DNMT1 in MPM H28 cells by a lentiviral-based RNA interference (RNAi) strategy. pLL3.7 lentiviral vector was used to constitutively express a DNMT1-targeted short hairpin RNA (shRNA) and almost 100% of viable H28 cells could be monitored (Fig. 2a).

After 6 h each cycle within 48 h. EGFP positivity of target cells was monitored to verify the efficiency of infection that approximately reached 90–95%. Stable EGFP expression was monitored for 1 month and, in the case of pLL3.7 and pLL3.7-p21sh1 infection, a negligible decrease of GFP positive cells was observed.

Cell cycle analysis was performed fixing the cells in ice-cold 70% ethanol solution (16 h at +4 °C) and resuspending them in propidium iodide staining solution (0.1% sodium citrate, 0.1% Triton X-100, 250 μg/ml RNase A, 50 μg/ml propidium iodide). Cytomfluorimetric acquisitions and sample analysis were performed by BD FACScan flow cytometer (BD Biosciences, San Jose, CA, USA) and FlowJo 8.6.3 software (Tree Star, Inc., Ashland, OR, USA), respectively.

Western blot analysis were performed as previously described [29].
DNMT1 silencing was confirmed by western blot analysis of H28 pLL3.7-MTsh1 infected cell lysate and, interestingly, no changes in p53 and p21 proteins expression were monitored (Fig. 2b) although the p21 transcripts were modestly increased (1.7 ± 0.19 folds respect to pLL3.7 infected H28 cells).

To better define the biological response of H28 cells, the cell cycle profile of DNMT1-depleted cells was monitored and compared with not infected and empty vector infected cells, observing a conspicuous amount of hypodiploid cells (49%) after 16 days of DNMT1 silencing, concomitantly with a genomic DNA fragmentation typical of programmed cell death (Fig. 2c and d). However, cell cycle analysis revealed only a slight increase of cells in S phase, an expected phenomenon since DNMT1 function is enzymatically exerted during DNA replication (Fig. 2b).

3.3. Decitabine induces decreased cell survival, cell cycle perturbation, cell death and modulation of p21 expression in MPM cells

In order to characterize the biological response of MPM cells to Decitabine treatment, we conducted a series of experiments by which cell survival, cell cycle alterations and apoptotic response of H28 cells were analyzed in both dose–response and time-course approaches. As reported in Fig. 3a, Decitabine effect on cell viability was detectable after 2 days of treatment starting at the concentration of 1 μM. Moreover, after 6 days, at Decitabine concentration of 1, 5, and 10 μM, the cell viabilities were reduced to less than 50%, respect to control cells.

The following studies of cell cycle profiles and apoptotic response were performed at the Decitabine concentration of 1 μM. Along with accumulation of variable percentage of hypodiploid cells (10–15%) after 6 days of treatment (Fig. 3b), important alterations of the cell cycle profile were monitored with an evident decreased of G1 phase and an increased in G2 phase percentages (Fig. 4d). In addition, H28 MPM cells, treated for 6 days with 1 μM of Decitabine, were not able to form colonies if replated, suggesting a not replicating capability of the residual viable cells (Fig. 3c).

In order to verify the involvement of either p53 and p21 tumor-suppressor genes in the MPM cellular response to Decitabine, western blot analysis were performed in both time-course and dose–response experiments observing an accumulation of p21, but not p53 protein, in a dose- and time-dependent fashion (Fig. 3d and e). Furthermore, a robust p21 transcripts accumulation was observed, by real-time quantitative PCR, after 6 days of 1 μM Decitabine treatment (12.34 ± 0.22 folds respect to untreated H28 cells). Com-
parable results were obtained in both MES-SS 01 and MES-PR 99 primary MPM cellular populations (data not shown).

3.4. Role of p21 upregulation in the biological response of MPM cells to Decitabine treatment

The role of p21 upregulation in the response of MPM cells to Decitabine was investigated in a lentiviral-based silencing approach. Through the same RNAi strategy used to silence DNMT1 expression, p21 protein synthesis was stably downregulated in H28 MPM cells that, later on, were treated with 1 μM of Decitabine for 6 days. Biochemical analysis confirmed the loss of p21 expression in pLL3.7-p21sh1 infected H28 total cell lysates (Fig. 4a). Interestingly, monitoring the cell viability in a series of time-course experiments, we first observed an evident reduction in the number of viable cells (about 50% respect to the control after 6 days) in p21-silenced H28 cells (Fig. 4b). Secondly, we found that the effect of Decitabine, in terms of decreased cell survival respect to control untreated cells, was partially reduced in p21-silenced H28 cellular population (Fig. 4c), suggesting an involvement of p21 in the mechanisms through which Decitabine exerts its antineoplastic activity. The role of p21 in the cellular response to Decitabine was also investigated pointing the attention on the perturbation of the cell cycle profile. Intriguingly, we found that the silencing of p21 was not able to inhibit the Decitabine-dependent G2/M arrest but, on the contrary, determined per se an increase of the cells accumulated in G2/M phase of the cell cycle (Fig. 4d).

4. Discussion

It is overall accepted that a normal cell must acquire multiple genetic abnormalities to definitely transform in a cancer cell. The variable period of time needed for cellular transformation seems to be shorter than expected because high genomic instability, determined by mechanisms that are still not completely understood, is thought to facilitate the accumulation of these genetic aberrations. However, the discovery of structurally altered heterochromatin loci in solid tumors, characterized by loss of DNA methylation, could explain, at least in part, this phenomenon.

Interestingly, the DNA methylation status of MPM has been investigated by other authors that analyzed 28 genomic loci and found the presence of highly methylated genes [1 1]. To better link possible alterations of the DNA methylation levels with the already reported high genomic instability in mesothelioma cells, we focused our attention first on the methylation status of MPM cells verifying a robust decrease of DNA methylation involving megabases sized clusters of pericentromeric sequences (satellite 2). Thus, DNMTs expression was monitored trying to find a mechanism
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Fig. 3. Decitabine treatment of H28 mesothelioma cells. (A) Cell survival of H28 cells in response to (♦), 0.1 μM; (□), 1 μM; (•), 0.5 μM; (▲), 10 μM of Decitabine treatments for 2, 4, and 6 days, reported as percentage respect to untreated cells. (B) Percentage of hypodiploid cells evaluated by propidium iodide staining at different time points at 1 μM of Decitabine treatment. (C) Colonies formation of two hundred H28 cells replated after treatment with 1 μM of Decitabine (DAC) for 6 days, untreated (nt) as control. (D and E) Western blot analysis of endogenous p53 and p21 expression in time-course (1 μM of Decitabine) and dose–response (6 days of treatment) experiments. (nt) untreated, and (DAC) Decitabine-treated H28 total cell lysates.

that could explain the reported loss of global DNA methylation. Paradoxically, in MPM cells all DNMTs (DNMT1, DNMT3a, and all isoforms of DNMT3b) appear more expressed, if compared with normal mesothelial cells, and probably involved in the local hypermethylation associated with decreased expression of tumor-suppressor genes. Moreover, as often happen in tumors, DNMT1 resulted the most abundant source of DNA methyltransferase activity in malignant mesothelioma cells. In the light of these observations, we compared the stable DNMT1 silencing and the treatment with demethylating agent (Decitabine), two strategies theoretically similar in the ability to reduce the DNA methylation and considered good candidates for future therapeutic strategies, in the same biological system (H28 cells). The biological response to DNMT1 silencing was monitored in H28 MPM cellular population founding a clear induction of programmed cell death, not associated with either relevant perturbation of the cell cycle or accumulation of p53 and p21 tumor-suppressor proteins, even if a slight accumulation of p21 transcripts was observed. These observations are in agreement with previous studies [25] that monitored decreased cell survival and apoptotic response of NSCLC, esophageal adenocarcinoma, and MPM cells, associated with modest upregulation of p21 transcripts, in response to DNMTs silencing.

Decitabine effects were investigated in the same cellular model performing several time-course and dose–response experiments that showed a decrease of cell survival and induction of a less dramatic apoptotic response. Similar biological alterations were monitored also in primary MPM cells (data not shown), confirming the antiproliferative activity of the demethylating drug tested. Interestingly, even after 6 days of Decitabine exposure, the cells were not able to proliferate as proved by the colony assay approach (Fig. 3c). This is an important point to discuss since Decitabine can be administered to patients only for short period of time, in consequence of the already monitored side effects and, fortunately, it seems to be anyhow sufficient to induce an irreversible biological effect, at least in vitro. This feature is even more important if considered in a contest of a possible co-treatment with other anticancer drug with which Decitabine has been demonstrated to synergise [32–34].

Differently from DNMT1 silencing, Decitabine is also able to arrest cells in G2/M phase of the cell cycle and to significantly increase the accumulation of p21 at both transcript and protein levels. The hypothetical role of p21 upregulation in Decitabine response has been investigated by silencing p21 expression in H28 MPM cells. The stable p21 downregulation, interestingly, induces a cellular arrest in G2/M phase and a reduced cellular proliferation of MPM cells. These phenomena could be explained with the apparent double activity of p21 protein that, if in one hand is able to exert the most known tumor-suppressor activity as CDK-inhibitor, in the
other hand seems to promote cyclin B-Cdc2 kinase activity in G2/M phase, acting as a proto-oncogene [35]. Interestingly, p21-silenced H28 cells showed a consistent reduction, but not a complete abrogation, of the response to Decitabine, in terms of cell survival, respect to control cellular populations. It is also necessary to consider that the lower replication rate of p21-silenced cells could decrease the incorporation of Decitabine into the genomic DNA inducing the refractoriety to Decitabine treatment. However, the more efficient response to Decitabine of pLL3.7 infected cells (after 4 days of treatment, Fig. 3a), respect to p21−/− cells (after 6 days of treatment, Fig. 4c) in a contest of comparable population doubling (Fig. 4b), indicates that a decreased Decitabine incorporation is not sufficient to explain the partial resistance observed in p21-silenced cells, suggesting an involvement of p21 protein in Decitabine activity. The partial protective activity of the p21 silencing suggests the possible involvement of other molecular mechanisms driving the Decitabine cellular response in addition to p21 upregulation. Moreover, it is not possible to completely dissect the role of p21 in the Decitabine response, by stable silencing strategies, because its expression is important to positive regulate the cell cycle progression. The effects of p21 silencing on the Decitabine-induced modifications of the cell cycle were also investigated, founding that the Decitabine-dependent arrest in G2/M phase is still monitorable and even more pronounced in p21-silenced cells.
This result strongly suggests that p21 upregulation is not involved in the G2/M block induced by Decitabine. Curiously, although Decitabine treatments determine a strong reduction of the DNMT1 protein levels (Fig. 4a), the biological response of MPM cells to this drug appears quite different respect that observed in the DNMT1 silencing experiments. These results suggest that the conspicuous decreased DNMT1 enzymatic activity in Decitabine-treated cells could be considered a secondary aspect of the biological response to the demethylating agent.

However, DNMT1 silencing remains a good therapeutic target in mesothelioma, and also in other kinds of tumor, for two reasons: first of all, solid tumors are characterized by DNMT1 protein upregulation, probably necessary to sustain both faster cellular proliferation and local DNA hypermethylation. Secondly, since DNMT1 silencing exerts its antiproliferative activity through a different mechanism respect to Decitabine, it can be considered an effective alternative (or also additional) approach for cancer treatment, especially for cases in which demethylating agents cannot be used.

Conflict of interest statement

There is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.jlungcan.2009.01.015.

References