Mad 1 Inhibits Cell Growth and Proliferation but Does Not Promote Differentiation or Overall Survival in Human U-937 Monoblasts

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Abstract
The Mad family proteins are transcriptional repressors belonging to the basic region/helix-loop-helix/leucine zipper family. They share a common obligatory dimerization partner, Max, with the oncoprotein c-Myc and antagonize the function of Myc to activate transcription. The Myc/Max/Mad network has therefore been suggested to function as a molecular switch that regulates cell growth and differentiation by controlling a common set of genes. To study the biological consequences of Mad1 expression for hematopoietic cell growth and differentiation, we used the U-937 monocytic differentiation model to generate cells with inducible Mad1 expression using the reversed tetracycline-controlled transactivator system. The elevated expression of Mad1 in these cells resulted in increased Mad1/Max heterodimer formation correlating with reduced expression of the Myc/Mad target gene ODC. Mad1-expressing U-937 cells in suspension culture proliferated slower and exhibited an increased number of cells in the G1 phase of the cell cycle. Further, growth in semisolid medium was almost completely inhibited. Mad1-expression, however, neither enforced spontaneous differentiation nor enhanced differentiation induced by the phorbol ester 12-O-tetradecanoylphorbol-13-acetate, retinoic acid (RA), or vitamin D3 but rather led to delayed RA-stimulated differentiation. Mad1-expressing cells were further found to be reduced in cell size in all phases of the cells cycle and particularly in response to RA-induced differentiation. Unexpectedly, whereas Fas-induced apoptosis was slightly attenuated in Mad1-expressing U-937 cells, Mad1 sensitized the cells to tumor necrosis factor-α-induced apoptosis. These results suggest that Mad1 primarily regulates cell growth and proliferation in these cells, whereas its role in cellular differentiation and survival seems to be more complex. (Mol Cancer Res 2004;2(8):464–76)

Introduction
The c-myc proto-oncogene is strongly implicated in tumorigenesis and plays an important role in the regulation of cell proliferation, differentiation, apoptosis, and other fundamental cellular processes (1, 2). It encodes a transcription factor of the basic region/helix-loop-helix/leucine zipper family and activates transcription through heterodimerization with the basic region/helix-loop-helix/leucine zipper protein Max. This obligatory partner enables specific Myc/Max binding to E-box DNA recognition sequences in target gene promoters (1, 2). In addition to Myc, Max also forms heterodimers with the basic region/helix-loop-helix/leucine zipper protein Mad/Mnt family including Mad1, Mxi1, Mad3, Mad4, and Mnt/Rox as well as with Mga (for review, see refs. 1, 3). In contrast to Myc, Mad/Mnt complexes repress transcription from E-box elements through recruitment of the repressor protein mSin3, which is part of a protein complex including the histone deacetylases HDAC1/HDAC2 (for review, see refs 1, 3). c-Myc, on the other hand, interacts with TTRAP, which is part of several SAGA-like complexes containing hGCN5, P/CAF, or Tip60 histone acetyl transferases (for review, see refs. 1, 2). This suggests that both Myc and Mad/Mnt regulate transcription at least in part through modulation of chromatin structure.

Studies of the expression of myc, max, and mad family genes in mouse and human tissues as well as in in vitro cell differentiation model systems suggest that most mad family genes are expressed in cells undergoing differentiation or in noncycling terminally differentiated cells, whereas the myc family genes are predominantly expressed in proliferative, undifferentiated cells (for review, see refs. 1, 3). Because Myc and Mad family E-box DNA recognition seem to be very similar if not identical (4–7), these findings suggest that c-Myc, Max, and Mad/Mnt form a network that functions as a molecular switch regulating a common set of genes. For instance, during differentiation, the ratio of Max-containing complexes shifts toward Mad1/Max predominance (1, 8), resulting in preferential Mad1 occupancy of the cyclin D2 and hTert Myc target gene promoters in vivo, correlating with HDAC recruitment, histone deacetylation, and repression of gene activity in differentiated cells (9, 10). An impressive number of c-Myc
target genes involved in multiple aspects of cell behavior, including cell cycle regulation, cell growth, metabolism, molecular synthesis, apoptosis, differentiation, senescence, adhesion, angiogenesis, genomic instability, etc., have been identified during recent years (for review, see refs. 1, 2). Although the studies of Mad/Mnt target genes have been less extensive, both overlapping and nonoverlapping target genes for Muc and Mad/Mnt have been reported (5-7, 9-12). Many of the nonoverlapping genes do not contain E-box sequences (12) and possibly involve alternative partners for Muc and Mad/Mnt, as indicated by the recently suggested role of the zinc finger protein Miz-1 in Muc-dependent repression of transcription via initiator-like sequences (2, 13).

In support of the Muc/Max/Mad network model, ectopic expression of Mad/Mnt inhibits cell proliferation and Muc/Ras cotransformation in several in vitro and in vivo systems (1, 3). These biological activities of Mad1 require the NH2-terminal Sin3 interacting domain (SID), suggesting that they involve transcriptional repression of target genes. Conversely, targeted disruption of mad, mxi1, or mnt in mice led to increased proliferative capacity in certain cell types, and the mxi1 and mnt knockout mice display increased tumorigenesis under certain conditions (14-16). c-Myc was recently shown to stimulate cell growth (size) independent of cell cycle status in certain cell types (17-19), whereas Mad1 was reported to reduce cell size in thymocytes (11). However, although c-Myc usually inhibits cell differentiation and promotes apoptosis (1, 2), there have been conflicting reports on the effects of Mad family genes on differentiation (11, 14-16, 20-23) and survival (11, 14, 16, 23-28).

Using the U-937 differentiation model system to study the regulation of mad family genes, we and others have shown that the expression of mad1 is most clearly correlated with monocyte differentiation among the mad/mnt family genes (8, 29, 30). We have used this model system to investigate the potential role of Mad1 during monocyte differentiation by generating clones with stable integration of an inducible mad1 construct in the reversed tetracycline-controlled transactivator (rtTA, tet-on) system. Our results show that Mad1-expressing U-937 cells exhibit retarded cell growth and accumulation of cells in the G1 phase of the cell cycle. However, Mad1 neither caused spontaneous differentiation nor enhanced induced differentiation but rather inhibited differentiation induced by retinoic acid (RA). Further, Mad1-expressing cells were smaller than control cells in all phases of the cell cycle and particularly during RA-induced differentiation. Surprisingly, Mad1 was also shown to sensitize the cells to tumor necrosis factor (TNF)-α-induced but not Fas-induced apoptosis.

Results
Establishment of an Inducible Mad1 Expression System in U-937 Cells
To study the biological effects of Mad1 expression in the U-937 differentiation model system, we used the inducible tet-on system (31) regulated by the rtTA-activating ligand doxycycline (Dox). Several stable clones containing the full-length Mad1 construct tetO-mad1 (U-937-TM clones) or the mutated version tetO-mad1ΔN lacking amino acids 1 to 56 including the SID domain (U-937-TMΔN clones) were selected and the presence of the constructs was confirmed by Northern blot analysis after treating the cells with Dox (data not shown). As controls, cells transfected with a tet operator construct lacking mad1 (U-937-TL clones) were selected.

Analysis of Mad1 protein synthesis in U-937-TM, U-937-TMΔN, U-937-TL, and parental clones was done by immunoprecipitation of [35S]methionine-labeled cell lysates with Mad1 antibodies followed by SDS-PAGE (Fig. 1A-C). The basal level of Mad1 expression was elevated in all TM-clones from moderate levels in clones TM9 and TM11 to high levels in clones TM2 and TM4 compared with the parental U-937-rTAT-A and U-937-1 cell lines and the U-937-TL1 control clone (Fig. 1A and C). These levels were as high or higher than the level of Mad1 in U-937 cells differentiated by 12-O-tetradecanoylphorbol-13-acetate (TPA; Fig. 1C), suggesting that the integrated tetO-mad1 constructs were “leaky.” Addition of Dox increased the expression to various degrees in all of the U-937-TM clones as well as in the U-937-TMΔN18 clone (Fig. 1A and B), and 0.5 to 0.8 μg/ml was found to be the optimal nontoxic level of Dox (data not shown). Kinetic analysis revealed a 3-fold increase in the Mad1 level in U-937-TM9 cells beginning from 6 hours after Dox treatment and continuing thereafter (Fig. 1C). The Mad1 level remained stable for at least 48 hours after induction (data not shown). Dox did not affect Mad1 expression in the U-937-TL1 control clone.

To investigate whether the increased level of Mad1 protein in the U-937-TM clones resulted in an increased amount of Max/Mad heterodimers, Max was immunoprecipitated under low stringency conditions, after which communoprecipitated Mad1 was released and reprecipitated with Mad1 antibodies. The amount of Mad1 in complex with Max as well as the total amount of Mad1 were substantially higher in the U-937-TM2 clone than in the U-937-TL1 control clone also in the absence of Dox and were further increased by Dox treatment (Fig. 1D). Dox treatment did not affect the amount of Mad1/Max complexes or total Mad1 in the U-937-TL1 control clone. Figure 1D also shows that similar amounts of Max were precipitated in the two clones.

To study if the elevated basal level and/or the Dox-induced expression of Mad1 influenced the expression of Myc/Mad target genes, we measured mRNA expression of ornithine decarboxylase (ODC), a known Mad1 target gene (1, 2, 32), by real-time reverse transcription-PCR (RT-PCR). Figure 1E shows that the basal ODC expression in Mad1-expressing U-937-TM9 cells was reduced to ~50% of the level in the U-937-TL1 control clone and was further reduced after Dox treatment. Transient transfection of an ODC promoter/luciferase reporter construct into U-937-TM11 cells showed a similar reduction in basal and Dox-regulated ODC promoter activity compared with the U-937 parental cells (Fig. 1F).

In summary, we have created U-937 clones that exhibit elevated Mad1 expression that could be further increased by Dox treatment. The increased level of Mad1 resulted in elevated levels of Mad1/Max heterodimers and correlated with reduced expression of the Myc/Mad target gene ODC.

3 Lars-Gunnar Larsson, unpublished results.
FIGURE 1. Generating an inducible Mad1 system in U-937 cells. **A** and **B**, Mad1 protein synthesis. The indicated tetO-mad1-containing U-937-TM clones (**A** and **B**), the parental U-937-rtTA-1 clone (**A**), and the tetO-mad1ΔN-containing U-937-TMΔN18 clone (**B**), were treated with 0.5 μg/mL Dox or left untreated for 16 hours and labeled with [35S]methionine. Cell lysates were immunoprecipitated with Mad1 antisera and analyzed by SDS-PAGE. **C**, Kinetics of Dox-stimulated Mad1 protein expression. U-937-TM9 cells were treated with Dox for the indicated time points and analyzed for Mad1 expression as in **A**. U-937-TL1 control cells treated with Dox (12 hours) and U-937-1 cells treated with TPA for 4 hours were used as negative and positive controls for Mad1 expression, respectively. **D**, Mad1/Max complex formation. [35S]methionine-labeled cell lysates from Dox-treated (16 hours) or untreated Mad1-expressing U-937-TM2 or U-937-TL1 control cells were immunoprecipitated by Max antisera under low stringency conditions. Coimmunoprecipitated Mad1 was released and immunoprecipitated with Mad1 antisera. As controls, Max and Mad1 were immunoprecipitated under high stringency conditions. **E**, Reduced expression of the ODC Myc/Mad target gene in Mad1-expressing cells. Total RNA was prepared from U-937-TM11 and U-937-TL1 cells in the presence or absence of Dox as indicated after which ODC mRNA expression was quantitated by real-time RT-PCR. **F**, Reduced ODC promoter activity in Mad1-expressing cells. U-937-TM11 and U-937-TL1 cells were transiently cotransfected with the ODC promoter/luciferase (Luc) reporter gene and CMV-β-gal. Promoter activity was measured by luciferase assays and normalized to β-gal activity. Columns, percentage of the level of ODC mRNA (**E**) or relative luciferase units (**RLU; F**) observed in untreated U-937-TL1 control cells.
Mad1-Expressing U-937 Cells Exhibit a Reduced Rate of Cell Proliferation

To investigate the phenotypic consequences of ectopic Mad1 expression, we studied the proliferative potential of U-937-TM cells. Two clones with high Mad1 basal levels of expression, U-937-TM2 and U-937-TM4, and two with moderate levels, U-937-TM9 and U-937-TM11, were seeded in suspension culture. As shown in Fig. 2A, the cell numbers of the Mad1-expressing U-937-TM clones were significantly lower than the numbers of the parental U-937-rtTA and the U-937-TL1 and U-937-TL4 control cells after 6 days in culture. This was not due to increased cell death (data not shown). The reduced proliferation of the U-937-TM clones contrasted to the unaffected proliferation rate of the U-937-TMΔN18 clone, suggesting that the NH2-terminus of Mad1, which contains the SID domain, is required for the antiproliferative action of Mad1 in agreement with previous reports (1, 3). Addition of Dox decreased the proliferation somewhat further in the Mad1-expressing clones, although some inhibition was observed also in the control clones. The small differences in growth rate between the different Mad1-expressing clones and the weak Dox effects might suggest that the basal expression of Mad1 in these clones may have exceeded a critical threshold level wherein proliferation was affected. Kinetic studies (Fig. 2B) showed that U-937-TM2 and U-937-TM9 grew at a much slower pace than the control clone U-937-TL1 both in the absence and in the presence of Dox throughout the 6-day experiment.

We next investigated whether the cell cycle distribution was affected in Mad1-expressing U-937 cells. Figure 3 shows that a higher percentage of Mad1-expressing U-937-TM2 and U-937-TM9 cells were positioned in the G1 phase and less cells in the S phase of the cell cycle both in the absence and in the presence of Dox following subculture in fresh medium compared with the control clone U-937-TL1. This was most evident 24 hours after subculture wherein 53% to 55% and 28% to 29% of the U-937-TM cells were in the G1 and S phases of the cell cycle, respectively, in which the corresponding number for the control clone was 42% and 46%. To study whether the higher number of Mad1-expressing cells in G1 was due to increased pausing/arrest in G1, we applied nocodazole, a cell cycle inhibitor that arrests cells in the G2-M phase of the cell cycle. Because G1 arrested cells will remain in G1 while cycling cells will accumulate in G2-M after nocodazole treatment, the former cells can be more clearly shown. Indeed, a 4- to 6-fold increase of Mad1-expressing U-937-TM cells in G1 phase was observed after treatment with nocodazole for 16 hours compared with U-937-TL1 control cells (Fig. 3, middle panels). Taken together, these results suggest that enforced Mad1 expression in U-937 cells in suspension results in growth retardation and partial accumulation in the G1 phase of the cell cycle but not in complete growth arrest and cell cycle arrest.

A dramatic difference in growth behavior was, however, observed when U-937-TM cells and U-937-rtTA cells were cultured in semisolid medium (Table 1). The efficiency of colony formation of U-937-rtTA cells was between 6% and 15% depending on the cell concentration, whereas the efficiencies of the U-937-TM clones were much lower and varied between 0% and 3%. These results suggested that the effect of Mad1 on cell proliferation was more pronounced at restrictive growth conditions.

Series A-Induced Differentiation Is Not Enhanced, but RA-Induced Differentiation Is Delayed in Mad1-Expressing U-937 Cells

We next investigated whether the stage of differentiation or the capacity to differentiate was altered in the U-937-TM expressing U-937-TM clones were significantly lower than the numbers of the parental U-937-rtTA and the U-937-TL1 and U-937-TL4 control cells after 6 days in culture. This was not due to increased cell death (data not shown). The reduced proliferation of the U-937-TM clones contrasted to the unaffected proliferation rate of the U-937-TMΔN18 clone, suggesting that the NH2-terminus of Mad1, which contains the SID domain, is required for the antiproliferative action of Mad1 in agreement with previous reports (1, 3). Addition of Dox decreased the proliferation somewhat further in the Mad1-expressing clones, although some inhibition was observed also in the control clones. The small differences in growth rate between the different Mad1-expressing clones and the weak Dox effects might suggest that the basal expression of Mad1 in these clones may have exceeded a critical threshold level wherein proliferation was affected. Kinetic studies (Fig. 2B) showed that U-937-TM2 and U-937-TM9 grew at a much slower pace than the control clone U-937-TL1 both in the absence and in the presence of Dox throughout the 6-day experiment.

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clones. Figure 4 (left panels) shows that the percentages of cells positive for the two monocyte/macrophage cell surface antigens CD11c and CD14 were not significantly altered in neither Mad1-expressing U-937-TM11 nor Mad1ΔN-expressing U-937-TMΔN18 cells compared with U-937-rtTA control cells irrespective of the absence or presence of Dox, suggesting that enforced expression of Mad1 did not result in spontaneous differentiation of U-937 cells. We cannot, however, at present rule out more subtle changes in the state of monocytic differentiation.

To address whether the Mad1-expressing cells exhibited an altered response to differentiation signals, the cells were stimulated with RA, vitamin D3 (VitD3), or TPA. Figure 4A shows that the percentage of CD11c-positive cells was substantially lower in the Mad1-expressing U-937-TM11 cells compared with U-937-rtTA and U-937-1 control cells after 3 days of RA stimulation both in the absence and in the presence of Dox. No reduction in CD11c positivity was observed in Mad1ΔN-expressing U-937-TMΔN18 cells, suggesting that the impaired CD11c expression required interaction of Mad1 with the Sin3 transcriptional repressor complex. However, by 5 days, the proportion of U-937-TM11 CD11c-positive cells was approximately the same as in the control cells. These results suggest that RA-induced differentiation was delayed in Mad1-expressing cells. Similar results were also found for other U-937-TM clones (data not shown). The corresponding experiments using VitD3 (Fig. 4B) and TPA (Fig. 4C) showed no significant changes among U-937-TM11, U-937-TMΔN18, and U-937 control clones in the expression of the differentiation antigens CD14 and CD11c irrespective of the presence or absence of Dox. Similar results with TPA, VitD3, and RA were obtained using suboptimal concentrations of the inducers (data not shown).

Table 1. The Growth of Mad-Expressing U-937-TM Clones and the Parental U-937-rtTA Clone in Semisolid Medium

<table>
<thead>
<tr>
<th>Clones</th>
<th>150,000 Cells/60 mm Plate</th>
<th>100,000 Cells/60 mm Plate</th>
<th>50,000 Cells/60 mm Plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>U-937-rtTA-1</td>
<td>21,375</td>
<td>15,105</td>
<td>3,135</td>
</tr>
<tr>
<td>U-937-TM9</td>
<td>475</td>
<td>190</td>
<td>0</td>
</tr>
<tr>
<td>U-937-TM11</td>
<td>4,465</td>
<td>1,425</td>
<td>2</td>
</tr>
</tbody>
</table>

NOTE: Culture in semisolid medium was done by seeding a 4 mL top layer of 1.5 × 10^5 to 0.5 × 10^5 cells/mL medium containing 0.3% agarose A and 0.8 μg/mL Dox on top of a 4 mL bottom layer of medium containing 0.6% agarose in 60 mm dishes. The number of colonies was scored after 10 days of culture. One representative experiment is presented.
U-937 cells exit the cell cycle and stop proliferating during differentiation. It was therefore of interest to determine whether the kinetics or amplitude of the proliferation arrest in response to differentiation signals was altered in Mad1-expressing U-937 cells. As shown in Fig. 4 (right panels), undifferentiated U-937-TM11 cells proliferated slower than U-937-TMΔN18 and U-937 control cells in agreement with Fig. 2. Figure 4B and C (right panels) shows that the cell numbers of TPA-differentiated and VitD3-differentiated U-937-TM11, U-937-TMΔN18, and U-937-rTA-1 cells were very similar at both 3 and 5 days.

**FIGURE 4.** Induced differentiation of Mad1-expressing U-937 cells. **A.** Mad1-expressing U-937-TM11, Mad1ΔN-expressing U-937-TMΔN18, and parental U-937-rTA-1 and U-937-1 cells were seeded at a concentration of 1.2 × 10^5 to 1.5 × 10^5 cells/mL and induced to differentiate with 1 μmol/L RA. Cell surface expression of the monocyte/macrophage antigen CD11c was measured by flow cytometry at the indicated time points. **B.** The cells were treated with 10^{-7} mol/L VitD3 (D3) with or without doxycycline after which the expression of the monocyte/macrophage surface marker CD14 was measured at the indicated time points as in A. C. The cells were treated with 1.6 × 10^{-7} mol/L TPA (T) for the indicated time points after which cell surface expression of CD11c was measured. Data from one representative experiment is presented.
after induction. Interestingly, RA-induced U-937-TM11 cells growth arrested faster than the RA-treated U-937-TMΔN18 and U-937 control cells as determined by the differences in cell numbers at days 3 and 5 after induction (Fig. 4A, right panels).

In summary, enforced expression of Mad1 neither induced spontaneous differentiation of U-937 cells nor changed the sensitivity to differentiation signals, with the exception of RA, wherein differentiation was delayed. This delay correlated with premature proliferation arrest in Mad1-expressing cells.

U-937 Cells Ectopically Expressing Mad1 Are Smaller Than Control Cells in All Cell Cycle Phases and During RA-Induced Differentiation

In some experimental systems, both c-Myc and Mad1 have recently been shown to affect cell size (11, 17-19). To investigate potential effects of Mad1 overexpression on cell size in our system, we measured the forward scatter of the cells by fluorescence-activated cell sorting. We observed that the Mad1-expressing U-937-TM11 cells were slightly smaller than the U-937-TL1 control cells, an effect that was enhanced by Dox treatment (Fig. 5A). To determine the cell size distribution in the different phases of the cell cycle, forward scatter was measured by fluorescence-activated cell sorting after staining the cells with Hoechst. The Mad1-expressing U-937-TM11 cells were smaller in all cell cycle phases, with a more prominent difference in the G1 and S phases of the cell cycle, compared with the U-937-TL1 control cells (Fig. 5B). Finally, we also compared the size of the cells during VitD3-induced and RA-induced differentiation. The Mad1-expressing U-937-TM6 cells were generally smaller than the U-937-rtTA control cells. The size difference was particularly pronounced in RA-differentiated cells treated with Dox (Fig. 5C).

In summary, Mad1-expressing U-937 cells were smaller than the control cells in all phases of the cell cycle, and this difference was particularly pronounced during RA-induced differentiation.

Mad1-Expressing U-937 Cells Are Sensitized to TNF-α-Induced but not Fas-Induced Apoptosis

To investigate whether Mad1 affected the response to apoptosis signals, the cells were treated with TNF-α, a well-known inducer of apoptosis (33). Figure 6A shows that the percentage of apoptotic cells, as determined by Annexin V staining, was higher and increased with faster kinetics in Mad1-expressing U-937-TM2 cells compared with U-937-TL1 control cells in response to TNF-α. Both these effects were elevated by Dox treatment. This increase in TNF-α sensitivity was not observed in Mad1ΔN-expressing U-937-TMΔN18 cells (Fig. 6B), suggesting that it involved Mad1-induced repression of target genes.

Because Mad1 has been reported to inhibit apoptosis induced by Fas and TRAIL, two other members of the TNF family, in osteosarcoma cells (28), we compared the apoptosis sensitivity of the Mad1-expressing U-937 cells to Fas agonist versus TNF-α. Figure 6B shows that, in contrast to the response to TNF-α, Mad1-expressing U-937-TM2 cells were somewhat less sensitive than U-937-TL1 control cells to Fas-induced apoptosis, whereas the opposite response was observed in Mad1ΔN-expressing U-937-TMΔN18 cells. Another notable difference in response to α-Fas antibody and TNF-α was that, whereas apoptosis induced by α-Fas antibody was effectively blocked by the cell permeable pan-caspase inhibitor Z-VAD-FMK, TNF-α-induced apoptosis was only partially blocked by this inhibitor in all three cell lines (Fig. 6B). TNF-α is reported to be able to induce both caspase-dependent and caspase-independent pathways of apoptosis and apoptosis-like cell death as well as necrosis in different cell types, sometimes simultaneously (34).

In summary, Mad1-expressing U-937 cells exhibited increased sensitivity to TNF-α-induced and somewhat decreased sensitivity to Fas-induced apoptosis.

Discussion

Based on the previous observations that Mad1 expression is frequently induced during differentiation processes (for review, see refs. 1, 3), we addressed what biological role Mad1 might play during this process by generating an inducible Mad1 tet-on system (31) based on the U-937 monocytic differentiation model. The elevated basal and increased Dox-inducible levels of Mad1/Max complexes as well as the reduced promoter activity and mRNA expression of the ODC Mad1 target gene in these cells suggest that the exogenous Mad1 proteins were active and played a functional role.

Phenotypic studies showed that Mad1-expressing U-937 cells proliferated substantially slower than the U-937 control cells. This was only partially enhanced by the ligand Dox likely due to the elevated basal expression of Mad1 that may have exceeded a threshold level effecting proliferation. Our results agree with previous reports showing Mad induced inhibition or retardation of cell proliferation and cell cycle progression in several cell systems such as astrocytoma, fibroblasts, glioblastoma, hematopoietic progenitor cells, hepatocellular carcinoma, osteosarcoma, preadipocytes, and thymocytes (11, 22, 24, 26-28, 35-38). This is also consistent with the increased proliferative capacity of cells from mad1−/−, mad1−/−, or mxi1−/− mice (14-16).

Cell cycle analysis of the Mad1-expressing U-937 cells showed an increased number of cells in the G1 phase of the cell cycle, most evident at lower cell concentrations (Fig. 5; data not shown). Experiments blocking the cells in G2-M by nocodazole showed that the Mad1-expressing clones contained a larger subpopulation of cells arrested in G1 than the control clone, although the majority of the Mad1 cells continued to cycle until the nocodazole block in G2. These results seem compatible with the view put forward by Hölz et al. (39) that Mad1 increases the probability of cells to arrest in (or exit the cell cycle at) G1 rather than affecting the duration of the cell cycle. To determine to what extent Mad1 enforces G1 arrest versus prolonged G1 phase duration in the U-937 system, it will be necessary to measure the cell cycle at the individual cell level. More pronounced accumulation in G1 in response to Mad1 was reported by Chen et al. (35) and Roussel et al. (36) in astrocytoma and NIH3T3 cells, respectively. Other groups using inducible mad1 systems in fibroblasts and osteosarcoma cells reported only smaller or no differences in the cell cycle distribution after induction of Mad1 during normal growth conditions (27, 28). In our study, the most dramatic consequence of Mad1 expression was shown during culture in semisolid medium, wherein colony growth was almost completely inhibited. This suggests that the effect of Mad1...
FIGURE 5. Mad1-expressing U-937 cells are reduced in cell size. A. The sizes of the Mad1-expressing U-937-TM11 (thick line) and the U-937-TL1 control cells (filled histogram) cultured in the absence (left) or presence (right) of 0.5 μg/mL Dox for 24 hours were determined by measuring forward scatter (FSC) using a FACScan. B. U-937-TM11 cells (thick line) and U-937-TL1 cells (filled histogram) were stained with Hoechst and separated into the G1 (left), S (middle), and G2-M (right) phases of the cell cycle after which the sizes of the cells were determined by measuring forward scatter as in A. C. Mad1-expressing U-937-TM6 cells (thick line) and U-937-rtTA-1 control cells (filled histogram) were left untreated (top panels) or induced to differentiate with VitD3 (middle panels) or RA (bottom panels) in the absence (left panels) or presence (right panels) of Dox for 24 hours after which the sizes of cells were determined as in A.
is most clearly manifested under restricted growth conditions. In agreement with previous results (1, 3), the NH₂ terminus of Mad1 containing the SID domain was required for the antiproliferative activity of Mad1 in U-937 cells, suggesting that it involves repression of Mad1 target genes via Sin3/HDACs.

There could be several different explanations why enforced Mad1 expression does not always result in robust G₁ cell cycle arrest. Because growth inhibitory or differentiation signals usually induce several mad family genes (22, 29, 40, 41), which are often coexpressed in differentiated tissues (40, 42), Mad1 may need to coordinate with other Mad family proteins. Another possibility is that the repressor activity of Mad1 is enhanced, for instance, through post-translational modifications of Mad1 itself, mSin3, or other components of the repressor complex, in response to antiproliferative signaling. Interestingly, the interaction between Sin3 and the SID-like domain of the Sp1 family repressor protein TIEG2 was recently reported to be disrupted by signaling (43). A third possibility is that Mad1 works in conjunction with other growth inhibitory molecules such as the cyclin-dependent kinase inhibitors or the retinoblastoma protein family members (for review, see ref. 44). In support of this hypothesis, the effects of targeted deletion of mad1 and mxi1 were enhanced in mad1/C₀/C₀/p27kip1/C₀ and mxi1/C₀/C₀/p16INK4a/C₀ double knockout mice, respectively (15, 45). It is also possible that moderate overexpression of Mad1 is not sufficient to fully shift the balance of the Myc/Max/Mad network under conditions where c-Myc is still expressed. Although we did observe repression of an ODC promoter/reporter construct and reduction of endogenous ODC mRNA expression in Mad1-expressing U-937 cells, a more extensive study of Myc/Mad target genes is required to answer this question. A fifth possibility is that Mad1 cannot fully overcome Myc because not all Myc targets genes are regulated by Mad. For instance, the cyclin-dependent kinase inhibitors p15INK4b and p21Cip1 that are repressed by Myc through Miz-1 (for review, see refs. 2, 13) do not seem to be targets for

FIGURE 6. Mad1-expressing U-937 cells are sensitized to TNF-α-induced but not Fas-induced apoptosis. A. Mad1-expressing U-937-TM2 and U-937-TL1 control cells were seeded at a concentration of 1 × 10⁵ cells/mL and treated with 5 or 20 ng/mL TNF-α in the absence or presence of 0.5 μg/mL Dox for 24 and 48 hours or left untreated as indicated. B. Mad1-expressing U-937-TM2, Mad1ΔN-expressing U-937-TMΔN18, and U-937-TL1 control cells were pretreated with 10 μmol/L Z-VAD-FMK or vehicle for 1 hour followed by 100 ng/mL Fas antibodies (for 24 hours), 10 ng/mL TNF-α, or no further treatment (for 48 hours) in the presence of 0.5 μg/mL Dox. Apoptosis was measured using an Annexin V-FITC staining kit.
Mad1. Recent studies in *Drosophila* showed both overlapping and distinct target genes for *Drosophila* Myc and the suggested orthologue to vertebrate Mad and Mnt, dMnt. Many of these distinct target genes may be under control of non-E-box sequences possibly through *Drosophila* Myc and dMnt DNA binding partners of other than dMax (12). These five explanations may not be mutually exclusive and could differ in importance depending on cell type or experimental conditions.

A high number of target genes for Myc (1, 2) and Mad (5, 11, 12) are involved in cell metabolism and macromolecular synthesis, suggesting that one important aspect of Myc and Mad function is to regulate the “cell growth” (size) aspect of cell proliferation. In certain but not all cellular settings, Myc seems to stimulate cell growth independent of the cell cycle, such as in B cells, *Drosophila* wings, hepatocytes, and T cells (17-19). Mad1, on the other hand, has been reported to reduce cell size in thymocytes (11). Our results show that enforced Mad1 expression can reduce cell size also in monocytes. The reduction of cell size was not simply due to accumulation of cells in the G1 because it was observed in all phases of the cell cycle (Fig. 5). The reason why Myc and Mad affect cell size independent of cell cycle phasing in some but not all cellular settings is unclear but may reflect differences in the coupling between cell growth and cell cycle control in different species and cell types as well as during neoplasia (for review, see ref. 46).

Enforced Mad1 expression in the U-937 system did not result in spontaneous differentiation and did not sensitize the cells to induced differentiation, rather RA-induced differentiation was delayed. The latter effect required the SID-containing NH2-terminal part of Mad1, suggesting that it involved transcriptional repression of genes by Mad1. These results contrast to those of Cultraro et al. (20), which suggested that enforced Mad1 expression induced differentiation in MEL cells. One possible explanation for this discrepancy is that Mad1 might affect differentiation only indirectly through its influence on cell proliferation. This may have different consequences for differentiation depending on cell type and stage of maturation. Usually, differentiation and proliferation are compatible at earlier stages but in conflict with each other at terminal stages of differentiation. For instance, U-937 cells proliferate actively during the first days of RA-induced differentiation prior to the G1 arrest. Interestingly, Mad1-expressing U-937 cells ceased proliferation prematurely and had reduced size in response to RA, correlating with delayed differentiation (Figs. 4 and 5). These results agree with observations by Pulverer et al. (22) suggesting that Mad1 interfered with adipocyte differentiation by inhibiting the proliferative burst, which is essential for the subsequent differentiation. Furthermore, some growth inhibitory signals for U-937 cells including transforming growth factor-β, which induces Mad1 expression in these cells,4 in fact inhibit differentiation (47). Queva et al. (26) reported that bone marrow cells from transgenic mice expressing Mad1 showed reduced *in vitro* colony-forming capacity and reduced cell numbers for both granulocyte/monocyte and erythroid lineages in response to cytokines compared with bone marrow cells from wild-type mice (26). Interestingly, the relative reduction was more pronounced for mixed granulocyte/monocyte and granulocyte colonies than for monocyte colonies. This resembles our results in that Mad1 preferentially affected the response to RA, which induces many granulocytic characteristics in U-937 cells, rather than the responses to TPA and VitD3, which induce typical monocyte/macrophage U-937 differentiation (29).

Similar to our results, enforced Mad1 expression did not significantly affect the morphology of the differentiated murine myeloid bone marrow cells, although the process was not studied kinetically (26). However, delayed thymocyte and embryonic developments were observed in Mad1 and Mnt transgenic mice, respectively, apparently due to reduced proliferation (11, 21). On the other hand, targeted deletion of *mad1* led to delayed terminal granulocytic differentiation presumably due to inhibited cell cycle exit (14). Our current hypothesis is therefore that the timing or fine-tuning of enforced Mad1 expression in the U-937 system may not favor RA-induced monocyctic differentiation, whereas it may have the opposite effect during MEL cell differentiation. An alternative explanation is that Mad1 somehow interferes with RA signaling. Because the Sin3/HDAC corepressor complex is used for transcriptional repression both by Mad/Mnt proteins and by the unliganded RA receptor/retinoid X nuclear receptors (for review, see ref. 48), interference between these systems is conceivable, but we have no evidence for this at present.

Taken together, these results favor a model wherein Mad/Mnt family genes primarily regulate cell growth and proliferation, whereas the effects on differentiation seem to be more indirect and dependent on the cellular context.

Mad1-expressing U-937 cells were sensitized to TNF-α-induced but not Fas-induced apoptosis, which was rather somewhat desensitized. The former may seem surprising because Myc is known to promote apoptosis induced by TNF-α (for review, see refs. 1, 2, 33). Mad1 have been reported to inhibit apoptosis induced by granulocyte/macrophage-colony stimulating factor withdrawal in hematopoietic progenitor cells (26) and by treatment with Fas, TRAIL, or UV irradiation in osteosarcoma cells (28). Further, decreased survival has been observed in some *mad1−/−, mad3−/−,* and *mnt−/−* cells in response to certain apoptotic stimuli (14, 16, 23). On the other hand, Mxi1 has been reported to induce apoptosis in glioblastoma cells (24), and Mad1 apparently promotes apoptosis induced by cCD3 in thymocytes (11) and by 1c-B in hepatocytes (25). There are also reports suggesting that Myc can inhibit apoptosis of cells in culture under certain conditions (25, 49-51). The common view that Myc and Mad are universally proapoptotic and antiapoptotic, respectively, may therefore be an oversimplification. The mechanism(s) by which Myc sensitizes cells to apoptosis is still unclear. Some proapoptotic E-box-containing Myc target genes such as *ODC, E2F, LDH-A, NRF-1, p53, Bax,* etc. (for review, see refs. 1, 2, 33), are or might potentially be repressed by Mad. Several observations, however, suggest that the proapoptotic function of Myc at least in part involves non-Mad targets possibly connected to repression of transcription by

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4 Wu et al., in preparation.
Myc (6, 52, 53). Our results show that both Mad-induced sensitization of TNF-α-induced apoptosis and desensitization of Fas-induced apoptosis in U-937 cells required the NH2-terminus of Mad1 containing the SID domain, suggesting that both responses involved repression of Mad1 target genes.

The mechanism by which Mad1 sensitizes U-937 cells to TNF-α-induced apoptosis (or desensitizes Fas-induced apoptosis) is currently unknown. Fas and TNF-α receptor 1 use a common signaling pathway to induce apoptosis involving FADD/caspase-8 and -10. Gehring et al. (28) reported that inhibition of Fas-induced apoptosis by Mad1 in osteosarcoma cells correlated with reduced induction of caspase-8 activity. However, TNF-α receptor signaling is much more complex than Fas signaling in that it involves both a proapoptotic pathway through FADD/caspase-8 and -10 and an antiapoptotic pathway via TRAF2/RIP1/nuclear factor-κB pathway, which can either inhibit or enhance apoptosis depending on the cell type or other circumstances (54). Given that Mad1 sensitized TNF-α-induced but not Fas-induced apoptosis in U-937 cells, it is conceivable that Mad1 inhibits the antiapoptotic nuclear factor-κB pathway or enhances the proapoptotic JNK pathway. This remains to be investigated.

In conclusion, our results suggest that ectopic expression of Mad1 in the U-937 system primarily affects cell growth and proliferation but does not promote differentiation or survival in general. The role of Mad1 in differentiation and survival may therefore be more complex, and its elucidation will require further investigation.

Materials and Methods

Cell Culture and Assays of Cell Growth, Differentiation, Cell Size, and Apoptosis

U-937 cells were cultured in RPMI 1640 supplemented with 10% FCS and antibiotics. The U-937 clone U-937-1 clone has been described previously (29, 30). U-937-rtTA-1, derived from U-937-1 cells, contains the rtTA transactivator (31), stably integrated, and was kindly provided by Dr. G. Öberg (Uppsala, Sweden). The Mad1-expressing U-937-TM, the Mad1ΔN-expressing U-937-TMΔN, and the control U-937-TL clones were generated from U-937-rtTA-1 cells as described below and contain stable integrations of the Dox-inducible constructs tetO-mad1, tetO-mad1ΔN, and tetO-luciferase, respectively.

Exponentially growing cells were induced to differentiate in medium containing 1.6 × 10^{-8} mol/L TPA (Sigma Chemical Co., St. Louis, MO), 10^{-7} mol/L VitD₃ (a generous gift from Hoffmann-La Roche, Basel, Switzerland), or 10^{-8} mol/L RA (Sigma Chemical) with or without 0.5 μg/mL Dox (Sigma Chemical). Differentiation was defined by the expression of the mature monocytic antigens CD11c, the α subunit of the gp105.95 adhesion molecule, and CD14, the receptor for lipopolysaccharide, visualized by immunofluorescence in a FACScan (Becton Dickinson, Mountain View, CA) using monoclonal antibodies LeuM5 and LeuM3 (Becton Dickinson), respectively, as described previously (30, 47).

Growth in suspension was measured from duplicate or triplicate cultures using a Coulter Z1 cell counter (Luton, England). Culture in semisoloid medium was done by seeding a 4 mL top layer of 25 × 10^{3} to 75 × 10^{3} cells/mL medium containing 0.3% agarose A (Pharmacia, Uppsala, Sweden) and 0.8 μg/mL Dox on top of a 4 mL bottom layer of medium containing 0.6% agarose into 60 mm dishes. The number of colonies was scored after 10 days of culture.

Cell size was assessed by measuring forward scatter of the cells by fluorescence-activated cell sorting. For cell cycle experiments, the cells were separated into different cell cycle phases by staining with 0.5 mg/mL Hoechst (Sigma Chemical) for 2 hours at 37°C. The sizes of the unfixed cells were assessed by measuring FCS.

For induction of apoptosis, 1 × 10^{5} cells/mL were seeded in the presence or absence of 5 to 20 ng/mL TNF-α (generously provided by Dr. G.R. Adolf, Ernst-Boehringer Institute, Vienna, Austria) or 100 ng/mL CD95-Fas antibody (Immunotech, Marseilles, France). To inhibit cellular caspase activity, cells were pretreated with 10 μmol/L of the cell permeable pan-caspase inhibitor Z-VAL-FMK (BD Biosciences PharMingen, San Diego, CA) for 1 hour before addition of apoptotic stimuli. Apoptosis was determined using Annexin V-FITC apoptosis detection kit I (BD Biosciences PharMingen) according to the manufacturer’s instructions.

Stable and Transient Transfections of U-937 Cells, Reporter Assays, and RT-PCR

Electroporation of U-937 cells for stable transfections were done as described (30). Linearized DNA (35 μg) was cotransfected with pRSV-hyg (5 μg), used as a selectable marker. Hygromycin (400 μg/mL, Calbiochem, San Diego, CA) was added 2 days after transfection, and resistant clones were picked 3 weeks after transfection, expanded, and analyzed for the presence of the integrated construct by PCR and Northern blot analyses. To generate the inducible constructs tetO-mad1 and tetO-mad1ΔN, a 1.1 kb NotI/EcoRI mad1 fragment from the full-length cDNA clone pVZ1mad1 (kindly provided by Dr. R.N. Eisenman, Seattle, WA) and a 0.5 kb BgII fragment from the plasmid CMVmadΔN encoding amino acids 57 to 222 of Mad1 (kindly provided by Dr. B. Lüscher, Aachen, Germany), respectively, were subcloned into the expression vector pUHD10-3, which contains tet operator sequences linked to a minimal promoter (31). The tetO-luciferase contains a luciferase fragment cloned into the expression vector pUHD10-3. The pUHD10-3 and tetO-luciferase constructs were kindly provided by Dr. M. Gossen (Heidelberg, Germany).

Transient luciferase and β-galactosidase reporter gene assays were done as described (30). The ODCΔLuc construct contains the ODC promoter, exon 1, intron 1 (containing two Myc-responsive E-boxes), and part of exon 2 in front of a luciferase reporter gene and was kindly provided by Dr. J.L. Cleveland (Memphis, TN).

For real-time RT-PCR, total RNA (4 μg) was converted to cDNA with RevertAid M-MulLV Reverse Transcriptase (Fermenta, Gothenburg, Sweden). ODC-specific primers and probe for RT-PCR were designed by the software Primer Express (Perkin-Elmer Applied Biosystems, Foster City, CA). As a reference, glyceraldehyde-3-phosphate dehydrogenase
was used. Sequences are provided on request. RT-PCR reactions were done using the Taqman PCR Core Reagent kit (Applied Biosystems, Foster City, CA) in triplicates with the ABI PRISM 7700 Sequence Detector (Perkin-Elmer, Foster City, CA).

Cell Cycle Assays

Cell cycle analysis was done as described previously (47). Briefly, $5 \times 10^5$ cells were harvested and washed once in cold PBS. The nuclei were prepared by treatment with 0.03 µg/mL trypsin (Sigma Chemical) for 10 minutes at room temperature followed by RNase A (Sigma Chemical) for 10 minutes at room temperature after which the nuclei were stained by 0.2 mg/mL propidium iodide (Sigma Chemical). The cells were allowed to incubate on ice in the dark for at least 15 minutes. Fluorescence intensity was measured using a FACScan (Becton Dickinson). The cell cycle profile was determined by using the MacCycle computer program (Becton Dickinson). To induce arrest in the G2-M phase of the cell cycle, cells were treated with 50 ng/mL of stringency precipitations, cells were lysed in AB and Tris lysis buffer, respectively (30), and immunoprecipitated with specific antibodies. An equal number of TCA-precipitable counts were used for each sample. The washing procedure for high and low stringency immunoprecipitations has been described (30). The samples were analyzed on 10% to 15% SDS-PAGE gels.

For detection of Myc, Mad1, and Max, IgG-7, a rabbit pan-Myc antisera (30), C-19 Mad1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), and C-17 Max antibodies (Santa Cruz Biotechnology) were employed.

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References


Mad 1 Inhibits Cell Growth and Proliferation but Does Not Promote Differentiation or Overall Survival in Human U-937 Monoblasts

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