Down-regulation of *MLL-AF9*, *MLL* and *MYC* expression is not obligatory for monocyte-macrophage maturation in AML-M5 cell lines carrying t(9;11)(p22;q23)

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Abstract. The *MLL-AF9* oncogene originates from the translocation t(9;11)(p22;q23), which is mainly associated with monocytic acute myeloid leukaemia (AML-M5; FAB-classification). In AML-M5 THP-1 cells carrying t(9;11)(p22;q23) and expressing *MLL-AF9*, we previously showed that *MLL-AF9* expression is down-regulated during monocyte-macrophage maturation. We have subsequently observed that in a 'rapid-growing' variant of the THP-1 cell line (THP-1-R) *MLL-AF9* down-regulation does not occur. MLL fusion proteins (including MLL-AF9) deregulate MYC transactivation activity, and both presence and absence of *MYC* down-regulation have been reported during monocyte-macrophage maturation in THP-1 cells. In the present study, we analyze the expression patterns of *MLL-AF9*, *MLL* wild-type and *MYC* after induction of monocyte-macrophage terminal differentiation in the AML-M5 cell lines, THP-1, THP-1-R, Mono-Mac-6 (MM6) and MOLM-13, all of which carry t(9;11)(p22;q23) and express *MLL-AF9*. RT-PCR analysis indicated that down-regulation of *MLL-AF9*, *MLL* or *MYC* is not necessary to abolish malignant phenotypes by induction of terminal monocyte-macrophage differentiation in leukaemic cells carrying t(9;11)(p22;q23).

Introduction

The *MLL-AF9* oncogene (1,2) originates from the translocation t(9;11)(p22;q23), which is mainly associated with monocytic acute myeloid leukaemia (AML-M5) (3). We recently showed that *MLL-AF9* expression is down-regulated during monocyte-macrophage maturation induced by ATRA (all-trans-retinoic-acid) or PMA/TPA (phorbol-12-myristate-13-acetate) in AML-M5 THP-1 cells (4) carrying t(9;11)(p22;q23) and expressing *MLL-AF9* (5). Since then, we have been surprised to observe that in THP-1 cells that have already undergone some months of continuous culture, PMA- or ATRA-induced differentiation is not accompanied by *MLL-AF9* down-regulation. Intriguingly, conflicting reports exist on the presence (6) or absence (7) of MYC down-regulation in THP-1 cells after PMA- or ATRA-induced differentiation. The *MYC* and *MLL* wild-type proto-oncogenes are both essential for normal myeloid proliferation and development (8,9). Naturally occurring MLL fusion proteins, including MLL-AF9, are potent transcriptional co-activators that induce a deregulation of MYC transcriptional factor and activate the transcription of promoters containing MYC E boxes (10; Galoian K, *et al*, Blood: abs. 457, 2000). The aim of the present study was to clarify the pattern of *MLL-AF9*, *MLL* and *MYC* expression after induction of monocyte-macrophage terminal differentiation in THP-1, MM6 and MOLM-13, all of which are AML-M5 cell lines carrying t(9;11)(p22;q23) and expressing *MLL-AF9* (5,11,12).

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Materials and methods

Cell lines. MOLM-13 cells were a generous gift from Dr Yoshinobu Matsuo (Fujisaki-Cell-Center, Okayama, Japan). MOLM-13, THP-1, and MM6 cells were cultured as described (4,12,13). Both types of THP-1 were differentiated with PMA (20 nM; Sigma, St. Louis, MO, USA) or ATRA (1 μM; Sigma), as previously reported (4). We have found that, like THP-1 cells, MOLM-13 cells are induced to stop proliferation and differentiate into mature macrophage-like cells within 24 h of treatment with PMA (20 nM). MOLM-13 cells had previously only been reported to differentiate to mature macrophage-like cells with interferon-γ, either alone or in combination with tumor necrosis factor-α (12). As regards MM6 cells after PMA-treatment (80 nM), approximately 50% of cells became adherent and differentiated (13). Accordingly, we have performed RT-PCR analysis only in adherent, non-proliferating MM6 cells, excluding cells still in suspension. For cell viability, cell aliquots were daily taken and counted in a haematocytometer; living cells were evaluated by trypan-blue dye exclusion test.

Table I. Oligonucleotide primers for PCR amplification.

Genes	Forward primers	Reverse primers	Product size (bp)
MLL-AF9 (THP-1)	AGCACTGGTCATCCCGCCTCAG	GGCAACAAGGTCCTTTGAGG	168
MLL-AF9 (MM6)	CTCCGGTCAATAAGCAGGAGAATG	TCGGCTGCCTCCTCTATTTACAG	320 and 434
MLL-AF9 (MOLM-13)	GAATCAGGTCCAGAGCAGAG	TCGGCTGCCTCCTCTATTTACAG	525 and 411
MLL	GAATCAGGTCCAGAGCAGAG	CTGAGGATGTTCAAAGTGCC	137
MYC	ACGAGGAGGAGAACTTCTACCAG	TTCACCATGTCTCCTCCCAGCAG	259
ß2M	CCTTGAGGCTATCCAGCGTA	TGGAGCAACCTGCTGCTCAGATA	522

Differentiation tests. Adherence: cells were considered to be adherent if they resisted vigorous shaking of the culture flask and washing by pipetting with normal growth medium. Phagocytosis and NBT (nitro blue tetrazolium reduction) tests: for phagocytosis, 1 μl of a 2.5% suspension of fluorescinated-latex-beads (2 μm-diameter; Sigma) was added to control and treated cells (5x10^5 cells/ml). For NBT-reduction, control and treated cells (5x10^5 cells/ml) were resuspended in 200 μl PBS-(phosphate-buffered saline) containing 0.1% NBT and 100 ng/ml PMA. In both tests, after incubation (37˚C for 60 min), the cells were stained with May-Grunwald-Giemsa and viewed using fluorescence microscopy (for phagocytosis; cells were also PBS-washed to remove excess beads) or light microscopy (for NBT). The percentage of cells with ingested latex-beads or containing blue black granules was examined by scoring at least 300 cells. CD analysis: the FITC-conjugated monoclonal antibodies CD3-(clone-SK7), CD4-(clone-SK3), CD5-(clone-L17F12), CD7-(clone-4H9), CD10-(clone-HI10a), CD11b-(clone-D12), CD14-(clone-MPP9), CD15-(clone-MMA), CD19-(clone-4G7), CD33-(clone-P67.6), CD41a-(clone-HIP8) and CD42a-(clone-Beb1) were purchased from Becton-Dickinson. Control and treated cells (1x10^6 cells/ml in PBS) were incubated with antibody at room temperature for 15 min, PBS-washed and analysed using a FACScalibur flow-cytometer (Becton-Dickinson).

RT-PCR analysis. Total RNA was isolated using the 'RNeasy-Mini-Kit' (Qiagen, Santa Clarita, CA, USA), according to the manufacturer's instructions. Reverse transcription (RT) for a total volume of 20 μl was performed using a Super-Script™ First-Strand Synthesis Kit (Gibco-BRL, Basel, Switzerland) according to the manufacturer's instructions. Standard polymerase chain reactions (PCRs) were performed using the primers listed in Table I. PCR conditions for MLL-AF9 amplification from MM6 cells were 4 cycles of 94˚C for 30 sec, 55˚C for 5 min and 68˚C for 10 min, followed by 35 cycles of 94˚C for 30 sec, 55˚C for 30 sec and 72˚C for 1 min, and finally one cycle of 72˚C for 6 min. PCRs were assayed on 2% agarose gel and stained with ethidium bromide (0.5 μg/ml). We recorded one MLL-AF9 amplification product in THP-1 cells (4), two in MM6 cells (11), and two in MOLM-13 cells (14). In all the cell lines examined, the housekeeping gene, ß-2M (ß-2 microglobulin), was constantly expressed at the same level. All experiments were repeated three times with similar results.

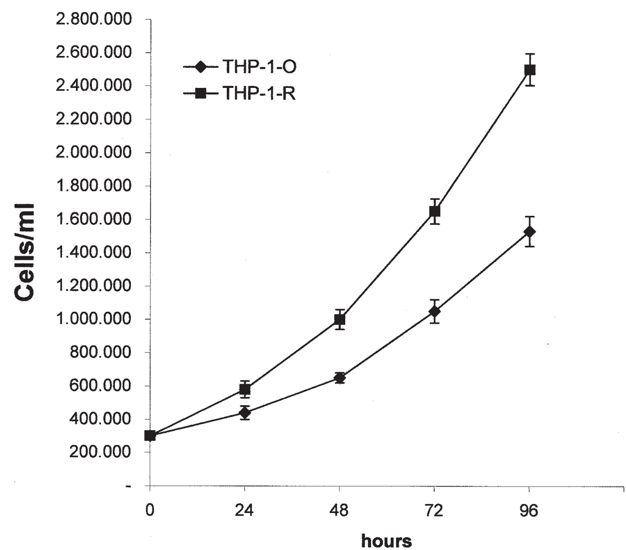

Figure 1. Proliferation of THP-1-O and THP-1-R cells. Number of viable THP-1-O and THP-1-R cells. The starting cell number was set at 3x10^5 cells/ml. Values represent the means ± s.d. of three separate experiments.

Results

As we have recently shown that *MLL-AF9* expression is down-regulated during monocyte-macrophage maturation in THP-1 cells (4), we have been surprised to observe that, in THP-1 cells that have already undergone some months of continuous culture, PMA- or ATRA-induced differentiation is not accompanied by *MLL-AF9* down-regulation. As long-term *in vitro* culturing can modify cells' original characteristics, we first compared phenotypic features (proliferation rate, morphology, adherence, phagocytosis, NBT reduction, and expression of surface antigens) of the THP-1 cells continuously cultured for over 3 months with those of cells newly thawed from liquid nitrogen. The only detected difference regarded proliferation rate, which was increased in the continuously cultivated cells (Fig. 1, Table II). This finding is consistent with a report that the original type of THP-1 cells [termed THP-1-O ('original')] and a THP-1 type continuously cultured after establishment [termed THP-1-R ('rapid')] retained the same phenotypic characteristics, except that THP-1-R cells showed a more rapid growth (15). After treatment with PMA or ATRA, *MLL-AF9* expression remained present in our differentiated

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Comparison of phenotypic characteristics between THP-1-O ('original' type) and THP-1-R cells ('rapid-growing' type).

A.

Characteristics	THP-1-O	THP-1-R
Morphology	Round, single cells in suspension	Round, single cells in suspension
Doubling time	ca. 48 h	ca. 24 h
NBT reduction (%)	3.2±0.3	3.7±0.4
Latex bead phagocytosis (%)	3.4±0.4	2.9±0.3
Antigen expression (%)		
Myeloid-lineage		
CD11b	10.4±1.7	11.2±1.2
CD14	2.4±0.4	2.7±0.6
CD15	91.2±4.2	89.7±4.7
CD33	95.4±4.0	96.2±3.7
Lymphoid-lineage		
CD3	2.7±0.4	3.1±0.3
CD4	93.0±4.1	91.6±3.9
CD5	3.7±0.7	3.2±0.9
CD7	64.8±7.2	62.6±6.7
CD10	0.8±0.2	0.6±0.2
CD19	0.8±0.1	0.9±0.3
MKE-lineage		
CD41a	3.3±0.6	2.7±0.5
CD42a	1.2±0.2	1.4±0.4

B.

	THP-1-O		THP-1-R	
Monocyte-macrophage maturation markers	+PMA 72 h (20 nM)	+ATRA 72 h (1 μM)	+PMA 72 h (20 nM)	+ATRA 72 h (1 μM)
Adherence and spreading	Present	Present	Present	Present
NBT reduction (%)	92.5±4.7	75.4±6.6	89.2±5.2	70.3±5.8
Latex bead phagocytosis (%)	27.2±2.7	23.3±2.3	28.6±3.1	25.1±2.6
CD11b expression (%)	77.2±7.2	65.5±5.4	72.3±6.2	61.2±5.1
CD14 expression (%)	26.1±3.1	21.0±2.1	25.6±2.8	19.5±1.9

Phenotypic characteristics of (A) untreated cells and (B) PMA- or ATRA-treated cells are shown. Values represent means ± s.d. of three separate experiments. CD4 and CD7 are ALL-T diagnostic markers, but they are also expressed in the myeloid lineage; MKE, megakaryocytic-erythrocytic.

THP-1-R cells until 72 h, whereas in THP-1-O cells no *MLL-AF9* expression was detected after either treatment (Fig. 2).

Successively, we have examined *MLL-AF9* expression in MOLM-13 and MM6, two other AML-M5 cell lines carrying t(9;11)(p22;q23) (11,12). In PMA-differentiated cells, *MLL-AF9* down-regulation was observed with MOLM-13 but not with MM6 cells, thus paralleling the results obtained with, respectively, THP-1-O and THP-1-R populations (Fig. 2). We have also monitored the *MLL* wild-type expression pattern, which resulted in being similar to the *MLL-AF9* expression pattern in all the cell lines tested (Fig. 2).

The *MYC* expression pattern in undifferentiated/differentiated THP-1, MM6, and MOLM-13 cells is shown in Fig. 2. Interestingly, *MYC* expression was down-regulated in PMA- or ATRA-differentiated THP-1-O cells, but not in PMA- or ATRA-differentiated THP-1-R cells. This observation could explain previous conflicting reports describing the presence (6) or the absence (7) of *MYC* down-regulation in PMA- or ATRA-differentiated THP-1 cells. In these reports, culture and growth features were not characterized. We also found that *MYC* expression was not down-regulated in PMA-differentiated MOLM-13 or MM6 cells.

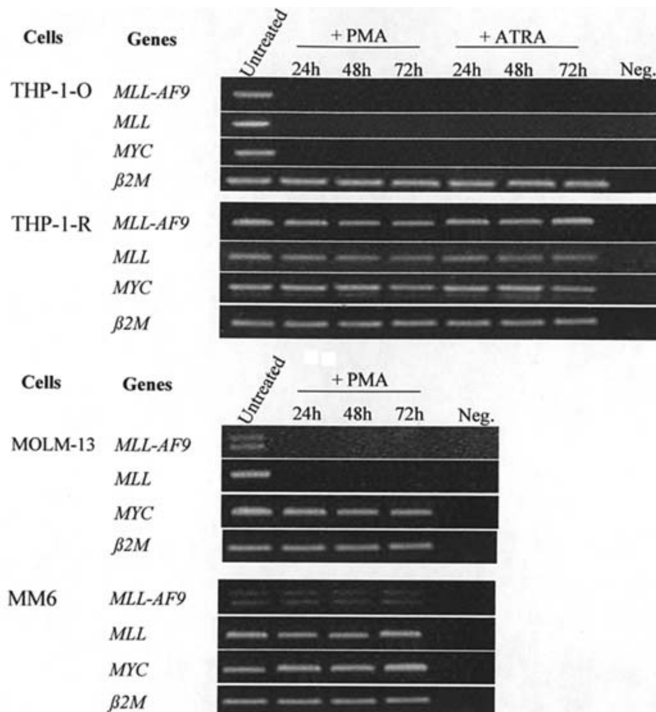

Figure 2. Expression of *MLL-AF9*, *MLL* and *MYC* in AML-M5 cell lines carrying t(9;11)(p22;q23) and differentiated along the monocyte-macrophage lineage. RT-PCR analysis of *MLL-AF9*, *MLL* and *MYC* expression time-courses in THP-1-O (i.e., 'original' type THP-1 cells), THP-1-R ('rapid-growing' type THP-1 cells that have been continuously cultured for several months), MOLM-13 and MM6 cells. We have detected a single MLL-AF9 amplification product in THP-1 cells (4), two in MM6 cells (12), and two in MOLM-13 cells (23). In all the cell lines examined, the housekeeping gene, ß-2M (ß-2 microglobulin), was constantly expressed at the same level. All the experiments were repeated three times with similar results.

Discussion

The data reported in this study support the hypothesis that *MLL-AF9* oncogene expression does not affect the monocyte-macrophage terminal differentiation process. In THP-1-O cells *MLL-AF9* expression was down-regulated upon monocyte-macrophage maturation induction (4). However, in THP-1-R, this down-regulation did not occur (Fig. 2), indicating that *MLL-AF9* expression down-regulation was not obligatory for the induction and maintenance of a state of terminal monocyte-macrophage differentiation of THP-1 cells. This result did not seem to be restricted to THP-1 cells. Indeed, *MLL-AF9* down-regulation was observed with MOLM-13 but not with differentiated MM6 cells (Fig. 2). Moreover, the terminal monocyte-macrophage differentiation induced by 1α,25-dihydroxyvitamin D3 in IMS-M1 AML-M5 cells, a line which also express *MLL-AF9*, does not require *MLL-AF9* down-regulation (Nagamura F, *et al*, Blood: abs. 587, 1995). In the light of these observations, *MLL-AF9* down-regulation does not seem to be necessary for the induction of monocyte-macrophage terminal differentiation in AML-M5 cells carrying t(9;11)(p22;q23). We have also assessed the *MYC* expression pattern and the findings indicated that down-regulation of *MYC* expression is also not required for the induction of monocyte-macrophage terminal differentiation in AML-M5 cells carrying t(9;11)(p22;q23).

It has been reported that *MLL-AF9* expression initially induces non-malignant expansion of myeloid precursors (2), with any perturbation of cellular differentiation being partial and largely reversible (16). Moreover, *MLL-AF9*-targeted down-regulation reduces THP-1 cell growth (4,17) without affecting terminal differentiation (4). MLL oncoproteins (including MLL-AF9) can deregulate MYC transactivation activity (10), and expression of *MYC* maintains myeloid proliferation without, *per se,* controlling myeloid differentiation (18,19). Moreover, in the leukaemic K-562 cell line treated with PMA, *MYC* expression is down-regulated, but its ectopic overexpression does not inhibit monocyte-macrophage differentiation (20).

These observations can be interpreted as evidence that *MLL-AF9* expression can cause elevated myeloproliferation by a mechanism that involves MYC without affecting terminal differentiation processes. Induction of terminal differentiation usually leads to down-regulation of the genes governing cell division, thereby interrupting proliferation. The data from the present study indicate that leukemic cells carrying t(9;11)(p22;q23) do not require down-regulation of the *MLL-AF9*, *MLL* and *MYC* genes (involved in myeloid proliferation) to abolish the malignant phenotype by induction of terminal monocyte-macrophage differentiation.

While the down-regulation of the expression of the *MLL-AF9*, *MLL* and *MYC* genes is not necessary for monocyte-macrophage maturation, further studies will be needed to determine if the block of myelo-monocytic differentiation, observed in leukemic cells expressing *MLL-AF9*, involves the suppression or the functional inhibition of the corresponding protein products.

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