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Matrix and serine protease expression during leukemic cell differentiation induced by aclacinomycin and all-*trans*-retinoic acid

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Abstract

In myeloid leukemia, immature leukemic cells are able to egress into peripheral blood to infiltrate extra-medullary organs. We therefore analyzed the migrating and invasive potential of human HL-60 and NB4 cell lines, representative of acute myelogenous leukemia, their ability to express matrix metalloproteases (MMPs), tissue inhibitors of metalloproteases (TIMPs) and urokinase plasminogen activator (uPA) in response to differentiating agents. Granulocytic differentiation by all-*trans*-retinoic acid (ATRA) and aclacinomycin (ACLA) strongly increased HL-60 and NB4 cell migration and invasion. At mRNA and protein levels, these cell lines produced significant amounts of MMP-9 (HL-60 < NB4). Granulocytic differentiation by ACLA increased both pro and active forms of MMP-9 whereas ATRA decreased them and stimulated uPA mRNAs. TIMP-1, the physiological MMP inhibitor, increased during granulocytic differentiation whereas TIMP-2 did not significantly vary. Use of Batimastat and aprotinin suggests that ATRA was active by modulating the uPA system while ACLA interfered with MMP expression. In conclusion, our data demonstrate that HL-60 and NB4 cells express MMPs and uPA which are differentially regulated by the differentiating agents ATRA and ACLA and suggest the clinical usefulness of MMPs and serine protease inhibitors in the prophylaxis and treatment of the ATRA syndrome.

Keywords: leukemia ; ATRA ; anthracyclines ; cell invasion ; MMPs ; serine proteases

Abbreviations: ACLA, aclacinomycin ; ACLA*, ATRA/ACLA combination ; ATRA, all-*trans*-retinoic acid ; BB-94, Batimastat ; ELISA, enzyme-linked immunosorbent assay ; MMPs, matrix metalloproteases ; NBT, nitroblue tetrazolium ; RT-PCR, reverse transcription-polymerase chain reaction ; TIMPs, tissue inhibitors of metalloproteases ; uPA, urokinase-type plasminogen activator.

1. Introduction

Abnormal proliferation and differentiation of immature white blood cells in the bone marrow generally characterize leukemia. In acute forms of myelogenous leukemia, immature leukemic cells are able to egress into peripheral blood and then infiltrate extra-medullary organs. These processes strongly contrast with normal hematopoiesis in which (i) only differentiated cells egress from the bone marrow and get access to the circulation and (ii) upon activation, granulocytes, monocytes or T cells migrate from the blood stream into extra-vascular tissues [1,2]. As shown by an increasing number of reports, it has become evident that the invasion process of leukemic cells is mediated by proteases including serine proteases and MMPs which degrade the basement membrane of the epithelial barrier and the underlying interstitial stroma [3,4]. MMPs are zinc-dependent endopeptidases secreted as proenzymes. Most of these enzymes are activated extra-cellularly by serine proteases or other MMPs [5]. Specific physiological inhibitors (TIMPs) inhibit these activated enzymes. The balance between activated MMPs and TIMPs determines the overall MMP proteolytic activity and consequently the extent of extra-cellular matrix turnover [6]. Among the different MMPs described so far, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) appear to be key proteases contributing to the degradation of basement membrane through their ability to cleave type IV collagen. MMP-2 activation is a membrane-associated mechanism requiring membrane-type MMPs (MT-MMPs) among which six different forms have been described [7,8].

It has been suspected that serine proteases and MMPs play an important role in the pathogenesis of hematological disorders such as inflammation and leukemia, particularly by contributing to the premature release of bone marrow blasts into peripheral blood and to cell dissemination. The steady-state production of blood cells depends to a large extent on the interaction between hematopoietic stem/ progenitor cells and the different

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components of the microenvironment present in the medullar cavity. Indeed, the expression and the role of cell surface adhesion molecules in cell-cell and cell-extra-cellular matrix interactions have been extensively studied in pathological hematopoiesis [9,10]. In contrast, little is known about the relationship between the invasive properties, the secretion of MMPs and the differentiation state of leukemic cells induced by drugs currently used in cancer therapy. To examine this relationship, human leukemic HL-60 and NB4 cell lines, representative of acute mylogenous leukemia, were induced to differentiate toward the granulocytic lineage by treatment with ATRA [11,12] and the anthracycline antitumor drug, ACLA.

Using these *in vitro* experimental models, we demonstrate that induction of granulocytic differentiation in HL-60 and NB4 cells leads to an increase of their *in vitro* migration and invasiveness, accompanied by a differential expression of MMPs and serine proteases, according to the inducer tested.

2. Materials and methods

2.1. Chemicals and reagents

The MMP inhibitor BB-94 [13] was from British Biotech (British Pharmaceuticals). The serine protease inhibitor aprotinin (Antagosan®) was from Hoechst Marion Roussel. Matrigel, a basement membrane preparation extracted from murine Engelbreth-Holm-Swarm tumors was prepared in our laboratory as previously described [14]. ATRA and ACLA were purchased from Sigma.

2.2. Cell culture

The human leukemic cells HL-60 [15] were purchased from American Type Culture Collection (CCL-240) and the NB4 cells [16] were obtained from Dr. Lanotte (Hôpital Saint Louis, Paris, France). The human fibrosarcoma HT-1080 cell line (CCL-121) [17] was used as an internal control for MMP expression. The cells were maintained in RPMI 1640 medium (Life Technologies) supplemented with 2 mM L-glutamine and 10% heat-inactivated fetal calf serum (FCS) (Life Technologies) under standard culture conditions. The cell viability and cell number were determined by the Trypan Blue dye-exclusion method.

For differentiation assays, exponentially growing HL-60 and NB4 cells were harvested by centrifugation then cultured at a density of 5×10^4 cells/mL in complete RPMI supplemented with the optimal inducing concentration of each drug for 3 days. Prior to treatment with ACLA, NB4 cells were pre-incubated for 30 min with ATRA 0.5 µM (ACLA*). Differentiation was assessed as the percentage of HL-60 and NB4 cells able to reduce NBT after acquisition of a phorbol ester-inducible respiratory burst response [18].

For gelatin zymography assays, serum-free cultures were performed as followed: HL-60 and NB4 cells in the exponential growth phase were washed in phosphate-buffered saline, seeded at 5×10^4 cells/mL in Ultra-CULTURE® medium (Biowhittaker) supplemented with 2 mM L-glutamine and treated with the differentiating agents. For HT-1080 cells, subconfluent monolayers of cells in 75 cm^2 culture flasks were washed twice with PBS and incubated with 4 mL of UltraCULTURE® medium. These cells constitutively secrete proMMP-9 (92 kDa) and proMMP-2 (72 kDa) [19]. Their treatment by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) for 24 hr increases proMMP-9 secretion and activates proMMP-2. Conditioned media and cells were harvested from these cultures and kept frozen at -20° until use.

2.3. Gelatin zymography

Gelatinolytic activities in conditioned media and cellular extracts were performed according to Heussen and Dowdle [20]. Frozen cells (10^4) were covered with 20 µL lysis mixture (0.1 M Tris-HCl pH 8.1, and 0.4% Triton X-100). An appropriate volume of conditioned media or cellular extract corresponding to 10^4 cells was applied to 10% SDS-polyacrylamide gels containing 1 mg/ mL gelatin (Sigma). After electrophoresis, SDS was removed from the gel by two incubations in 2% Triton X-100 for 30 min. After overnight incubation at 37° in TCS buffer (50 mM Tris-HCl pH 7.4, 0.2 M NaCl and 5 mM $CaCl_2$), the gels were stained for 90 min with Coomassie Blue. Proteolytic activities were evidenced as clear bands against the blue background of stained gelatin. In some experiments, 10 mM EDTA were added to the TCS buffer in order to assess the MMP nature of the gelatinolytic activities.

2.4. Reverse transcription-polymerase chain reaction analysis

Total RNA was extracted from the two leukemic cell lines by RNA Instapure® treatment (Eurogentec). RT-PCR was performed on 10 ng of total RNA in a final volume of 25 µL using a Perkin-Elmer kit and following manufacturer's instructions. The sequences of the primers (Eurogentec) introduced in this study are given in Table 1.

Reverse transcription was carried out during 15 min at 70°. PCR conditions were 9572 min, followed by 30 cycles consisting of 94720, 66720 and 72730 s and a final elongation step of 7272 min. To control the efficiency of the RT-PCR, we designed a synthetic RNA, which can be reverse transcribed and amplified with the same

primers. This internal control RNA template, pCTR01 was added in each sample. The amplification products were electrophoresed on a polyacrylamide gel, stained with Gelstar (Sanver Tech.), scanned with a fluorS-Imager, and analyzed using multianalyst software (Bio-Rad).

Table 1 *Oligonucleotides primers*[a]

Oligonucleotides	Endogen (bp)	pCTR01 (bp)	Copies per 10 ng
MMP-2			
5'-GCGGAGATTGGGAACCAGCTGTA-3' (forward)	225	271	5×10^4
5'-GACGCGCCTGTGTACACCCACA-3' (reverse)			
MMP-3			
5'-GATCTCTTCATTTTGGCCATCTCTTC-3' (forward)	246	272	2×10^3
5'-CTCCAGTATTGTCCTCTACAAAGAA-3' (reverse)			
MMP-9			
5'-AGATCTTCTTCTTCAAGGACCGGTT-3' (forward)	208	266	100
5'-GGCTGGTCAGTGGCTTGGGGTA-3' (reverse)			
MMP-11			
5'-ATTTGGTTCTTCCAAGGTGCTCAGT-3' (forward)	155	268	10^3
5'-CCTCGGAAGAAGTAGATCTTGTTCT-3' (reverse)			
MMP-12			
5'-ACATTTCGCCTCTCTGCTGATGAC-3' (forward)	261	202	25×10^3
5'-CAGAAACCTTCAGCCAGAAGAACC-3' (reverse)			
MT1-MMP			
5'-GGATACCCAATGCCCATTGGCCA-3' (forward)	221	269	3×10^3
5'-CCATTGGGCATCCAGAAGAGAGC-3' (reverse)			
MT2-MMP			
5'-CTTGCAGAGATGCAGCGCTACTAC-3' (forward)	266	208	3×10^3
5'-CTGGATGCTAAAGGTCAGATGGTG-3' (reverse)			
MT3-MMP			
5'-GGTTGGATTTCGTGCATCATTCGG-3' (forward)	266	215	3×10^3
5'-ATAGAACTGCTGCATGGCAGCTAG-3' (reverse)			
UPA			
5'-ACTACTACGGCTCTGAAGTCACCA-3' (forward)	261	205	2×10^5
5'-GAAGTGTGAGACTCTCGTGTAGAC-3' (reverse)			
GAPDH			
5'-CCTGGCCAAGGTCATCCATGACA-3' (forward)	183	262	2×10^3
5'-GGGATGACCTTGCCCACAGCCTT-3'(reverse)			

[a] For each case, the expected sizes of the endogenous and the pCTR01 bands are indicated in columns 2 and 3, respectively. The number of internal control RNA templates introduced in each RT-PCR to monitor the assays are indicated in column 4.

2.5. Invasion and migration assay

In vitro invasion was determined in the matrigel-based assay as described previously by Janiak *et al.* [21]. Briefly, 13 mm polycarbonate filters of 8 μm pore size (Costar) were coated with 50 μg matrigel. The lower compartments of the transwell chambers were filled with serum-free UltraCULTURE® medium. Leukemic cells suspended in UltraCULTURE® medium were placed in the upper compartments (4×10^5 cells per chamber) and incubated for 6 hr at 37°, in 5% CO_2. Following incubation, cells that had migrated through the matrigel-coated filters were recovered from the lower compartments and counted. Percentage of invasion was calculated by considering the number of cells in the initial cell suspension as 100%. In order to inhibit MMPs or plasmin during the invasion assay, leukemic cells differentiated or not, were incubated with BB-94 (1 μM) or aprotinin (0.5 μg/mL). At these concentrations, cell viability was not affected as assessed by the Trypan Blue staining. Results are expressed as percentage of invasion inhibition. To study cell migration, filters were not coated with matrigel and subsequent procedure was the same as for the invasion assay. Each experiment was performed in triplicate for each sample, and repeated at least three times.

2.6. Enzyme-linked immunosorbent assay

MMP-9, MMP-2, TIMP-1 and TIMP-2 proteins were quantified in conditioned media (corresponding to 10^5 cells), measured by ELISA (Biotrak™, Amersham) according to the manufacturer's instructions.

2.7. Statistical analysis

Our data represent the mean value ± SD of six independent experiments conducted in triplicate. Parameters were evaluated using Student's *t*-test. The level of significance chosen was 95% ($P < 0.05$).

3. Results

3.1. Induction of differentiation of HL-60 and NB4 cells

We first determined the optimal concentration of ACLA and ATRA required for the differentiation of HL-60 and NB4 cells. As shown in Table 2, treatment with ATRA (1 μM) and ACLA (30 nM) induces HL-60 cell differentiation towards the granulocytic lineage, with 65 and 71% of NBT-positive cells, respectively. NB4 cells undergo granulocytic differentiation after treatment with ATRA 1 μM (76% of NBT-positive cells). Pre-exposure of NB4 cells to a low concentration of ATRA (0.5 μM) was required to induce differentiation with ACLA (25 nM) (81% of NBT-positive cells) (ACLA*).

When used at their optimal inducing concentrations, differentiating agents did not affect cell viability as assessed by Trypan Blue exclusion assay (<5% of dead cells). In addition, ATRA exhibited a growth inhibitory effect on HL-60 and NB4 cells (45 and 37%, respectively). On the contrary, the antitumor drug ACLA drastically inhibited the growth rate of the two cell lines tested (74-80%).

Table 2 *Effect of differentiating agents on the growth and differentiation of HL-60 and NB4 leukemic cells*

Inducer	Cell line	Optimal inducing concentration[a]	Differentiated cells (%)[b]	Growth inhibition (% of control)
ATRA	HL-60	1 μM	65 ± 4	45 ± 4
ACLA		30 nM	71 ± 2	75 ± 3
ATRA	NB4	1 μM	76 ± 2	37 ± 3
ACLA*[c]		25 nM	81 ± 7	74 ± 6

[a] Cells in exponential growth phase were treated with the optimal inducing concentration of each drug for 3 days. [b] The percentage of differentiated cells was determined on day 3 using nitroblue tetrazolium test for HL-60 and NB4 cells. It never exceeded 5% in control cells. [c] For NB4 differentiation with ACLA (25 nM), a pre-exposure of cells with ATRA (0.5 μM) for 30 min was required prior to treatment with the anthracycline.

3.2. Effect of differentiating agents on leukemic cell migration and invasion

Only a small fraction of HL-60 cells (1.3%) were able to migrate across porous, uncoated polycarbonate filters in a 6 hr assay. In the same conditions, the migration rate of NB4 cells was significantly higher (4.5%; $P < 0.02$ by comparison to HL-60 cell invasiveness), $n = 4$; error bars = SEM (Table 3). Both granulocytic inducers dramatically stimulated migration of HL-60 cells, with a proportion of migrating cells of 14 and 29%, after ATRA and ACLA treatment, respectively. A similar effect was observed with NB4 cells in which 12 and 26% of cells migrated after ATRA and ACLA* treatment, respectively. For invasion assays, the polycarbonate filters were coated with the reconstituted basement membrane matrigel. As shown in Table 3, the proportion of uninduced cells passing through the matrigel layer was lower or similar to that of cells crossing the uncoated filters. As observed with cell migration, granulocytic differentiation strongly increased cell invasiveness of HL-60 and NB4 cells. This enhanced cell invasion was time-dependent and already appeared after 1 day of treatment with 7.5 and 6% of invasive ATRA- and ACLA-treated HL-60, respectively, vs. 1% in control cells (data not shown).

3.3. Effect of Batimastat on leukemic cell invasion

We tested the effect of a synthetic MMP inhibitor, Batimastat, on cell migration and invasion of HL-60 and NB4 following ATRA, ACLA or ACLA* treatment. BB-94 (1 μM) was added to the upper compartment of the chamber during the assay. Although BB-94 did not affect cell migration, it significantly inhibited the invasion of the differentiated HL-60 and NB4 cells (Table 4). Both invasive HL-60 and NB4 cells were reduced in number after ATRA and ACLA treatment by 44.5 and 51%, and 24.5 and 50%, respectively.

Table 3 *Effect of differentiating agents on migration and invasiveness of HL-60 and NB4 leukemic cells*[a]

Inducer	Cell line	Cell migration (%)	Cell invasiveness (%)
Control	HL-60[b]	1.3 ± 0.3	1.0 ± 0.2
ATRA 1 µM		14.0 ± 4.0	10.0 ± 2.1
ACLA 30 nM		29.0 ± 3.0	22.0 ± 5.0
Control	NB4[c]	4.5 ± 1.1	3.0 ± 0.9
ATRA 1 µM		12.0 ± 1.8	13.7 ± 3.3
ACLA* 25 nM		26.0 ± 2.4	19.6 ± 1.7

[a] On day 3, cell migration and invasion were evaluated in migration chambers for 6 hr. Results are expressed as percentage of migration and invasion as indicated in Section 2. Results are the mean values of six experiments with triplicated wells ± SD.
[b] $P < 0.05$ by comparison between treated and control HL-60 cells.
[c] $P < 0.05$ by comparison between treated and control NB4 cells.

Table 4 *Effect of Batimastat on migration and invasiveness of HL-60 and NB4 leukemic cells*[a]

Inducer	Cell line	Cell migration (%)		Cell invasiveness (%)	
		Without Batimastat	With Batimastat	Without Batimastat	With Batimastat
Control	HL-60	2.2 ± 0.2	2.1 ± 0.3	1.6 ± 0.2	0.9 ± 0.2[b]
ATRA 1 µM		14.5 ± 3.6	14.1 ± 3.2	11.7 ± 1.6	6.5 ± 1.3[b]
ACLA 30 nM		29.0 ± 3.0	28.0 ± 3.2	22.5 ± 2.0	11.2 ± 1.4[b]
Control	NB4	4.6 ± 1.2	4.5 ± 1.1	3.0 ± 0.7	1.5 ± 0.4[c]
ATRA 1 µM		14.0 ± 1.6	13.5 ± 1.8	11.5 ± 1.9	8.8 ± 3.0[c]
ACLA* 25 nM		25.7 ± 2.2	25.3 ± 1.9	19.8 ± 1.5	10.0 ± 2.7[c]

[a] On day 3, leukemic cells differentiate or not were incubated with Batimastat 1 µM in migration chambers for 6 hr. The percentage of migrating and invading cells was estimated as indicated in Section 2. Mean value of six experiments with triplicated wells performed in each, ±SD.
[b] $P < 0.02$ by comparison between treated and control HL-60 cells.
[c] $P < 0.03$ by comparison between treated and control NB4 cells.

3.4. Effect of differentiating agents on the expression of MMPs

Competitive RT-PCR analysis, gelatin zymography, and ELISA documented the expression and production of MMPs that could promote cell invasion in cells induced or not to differentiate (Figs. 1 and 2). NB4 cells expressed and secreted a high level of MMP-9 (31 ng/10^5 cells), compared to HL-60 cells (5.5 ng/10^5 cells). MMP-2 was absent from HL-60 cells and weakly expressed in NB4 cells. Interestingly, in the two cell lines, granulocytic inducers such as ATRA and ACLA which increased cell invasion, exhibited a differential effect on MMP-9 expression. Indeed, ATRA decreased MMP-9 transcripts and protein in both cell types and ACLA or ACLA* markedly enhanced MMP-9 gelatinolytic activity and production. In addition, except with ATRA, zymographic analysis of cellular extracts demonstrate that these two myeloid cell lines secrete an active form of MMP-9 (83 kDa) (Figs. 1B and 2B). We also tested by competitive RT-PCR, the expression of other members of the MMP family such as stromelysins (MMP-3, MMP-11), metalloelastase (MMP-12) and membrane-type metalloproteases (MT-MMP-1 to -3). Among these proteases, only MT2-MMP mRNA was identified in NB4 cells and granulocytic differentiation with ACLA* stimulated MT2-MMP expression (Figs. 1A and 2A).

3.5. Effect of differentiating agents on the expression of TIMPs

Given the importance of TIMPs regarding the control of MMP activity, we studied whether undifferentiated or differentiated leukemic cells (Fig. 3A and B) expressed these inhibitors. NB4 cells secreted 10-fold more TIMP-1 than HL-60 cells (30 vs. 3 ng/10^5 cells). Both inducers increased TIMP-1 production with no significant variation of TIMP-2.

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Fig. 1. *Expression of MMP-2, MMP-9, MT1-MMP and MT2-MMP by HL-60 cells induced or not to differentiate.* (A) Competitive RT-PCR analysis of MMP-2, MMP-9, MT1-MMP, MT2-MMP and GAPDH was performed on total RNA. Endogenous MMP-2, MMP-9, MT1-MMP, MT2-MMP and GAPDH were detected at their expected sizes (225, 208, 221, 208 and 183 bp, respectively). pCTR01 control bands for MMP-2, MMP-9, MT1-MMP, MT2-MMP and GAPDH were obtained at the expected sizes 271, 266, 269, 266 and 262 bp, respectively. Proteases were expressed as a ratio to their specific internal control. The levels of mRNAs were normalized with GAPDH. (B) Zymographic analysis of conditioned media and cellular extracts of HL-60 cells induced or not to differentiate with the optimal concentration of each drug for 3 days. Medium conditioned by HT-1080 cells was used as control. (C) MMP-9 and MMP-2 ELISA of media conditioned by HL-60 cells induced or not to differentiate; base pairs (bp).

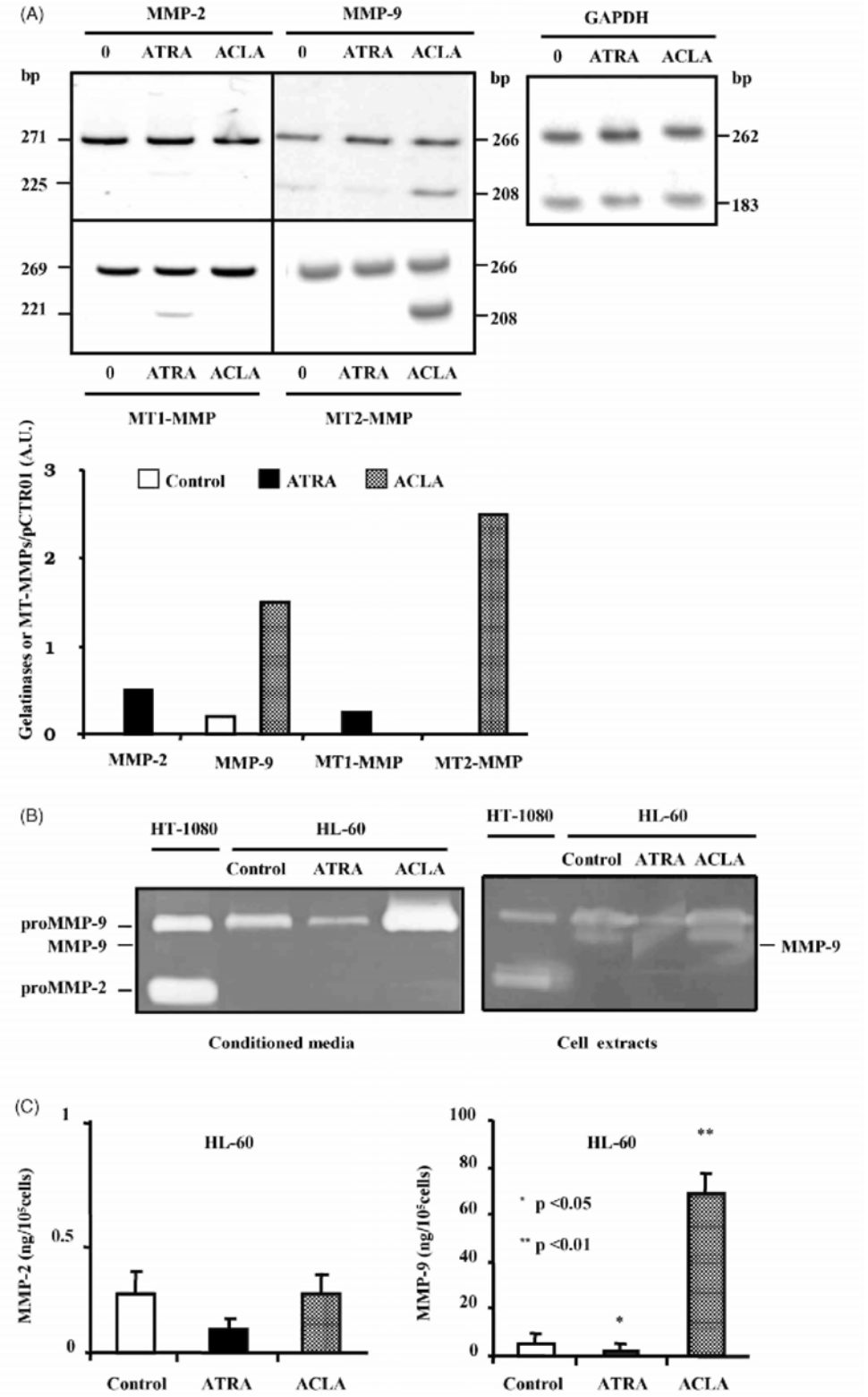

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Fig. 2. *Expression of MMP-2, MMP-9, MT1-MMP and MT2-MMP by NB4 cells induced or not to differentiate.* (A) Competitive RT-PCR analysis of MMP-2, MMP-9, MT1-MMP, MT2-MMP and GAPDH was performed on total RNA. Endogenous MMP-2, MMP-9, MT1-MMP, MT2-MMP and GAPDH were detected at their expected sizes (225, 208, 221, 208 and 183 bp, respectively). pCTR01 control bands for MMP-2, MMP-9, MT1-MMP, MT2-MMP and GAPDH were obtained at the expected sizes 271, 266, 269, 266 and 262 bp, respectively. Proteases were expressed as a ratio to their specific internal control. The levels of mRNAs were normalized with GAPDH. (B) Zymographic analysis of conditioned media and cellular extracts of NB4 cells induced or not to differentiate with the optimal concentration of each drug for 3 days. (C) MMP-9 and MMP-2 ELISA of media conditioned by NB4 cells induced or not to differentiate.

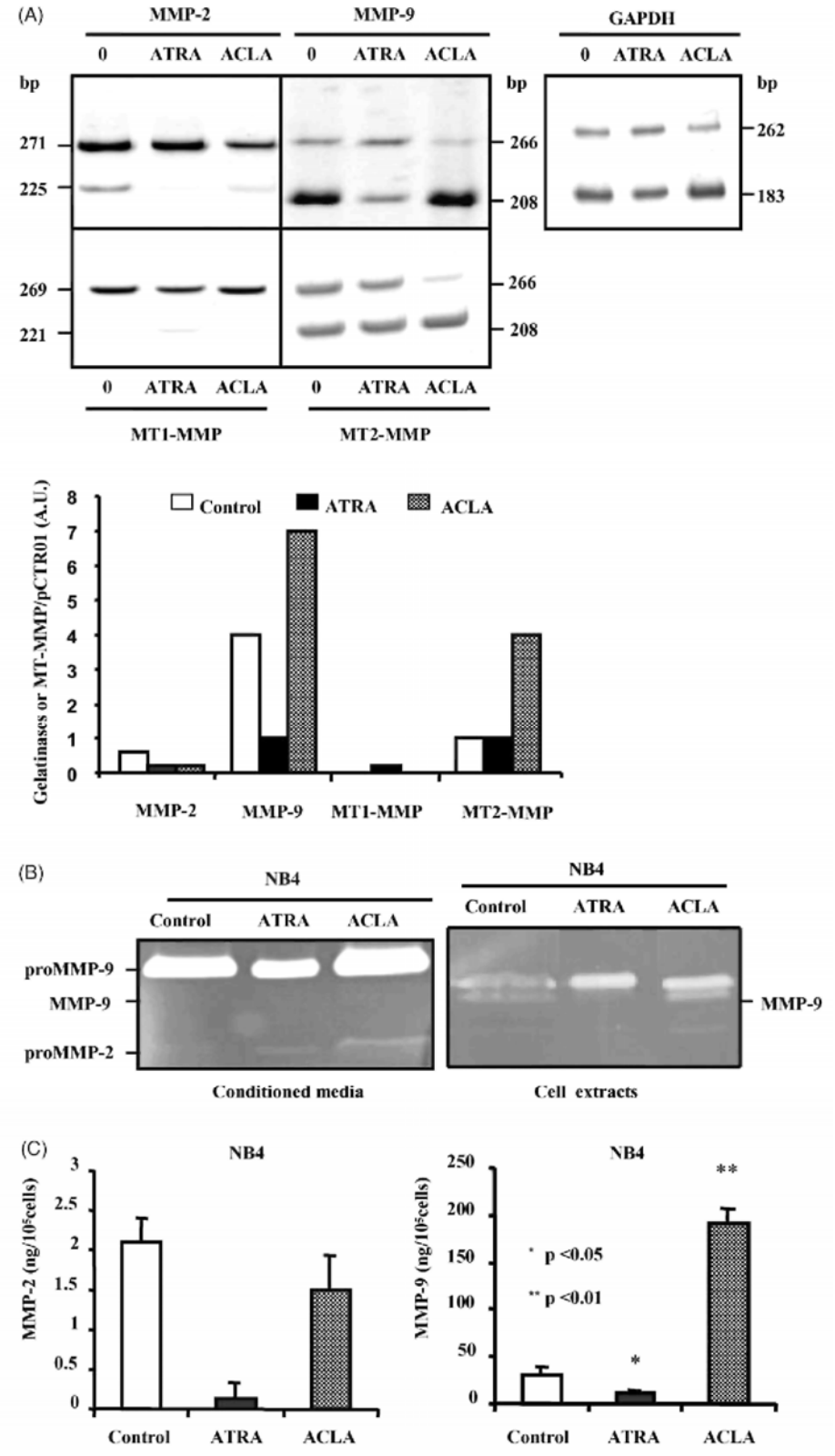

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Fig. 3. *Expression of TIMPs during induction of differentiation of HL-60 and NB4 cells. TIMP-1 and TIMP-2 ELISA of media conditioned by HL-60 (A) and NB4 (B) cells induced or not to differentiate with the optimal concentration of each drug for 3 days.*

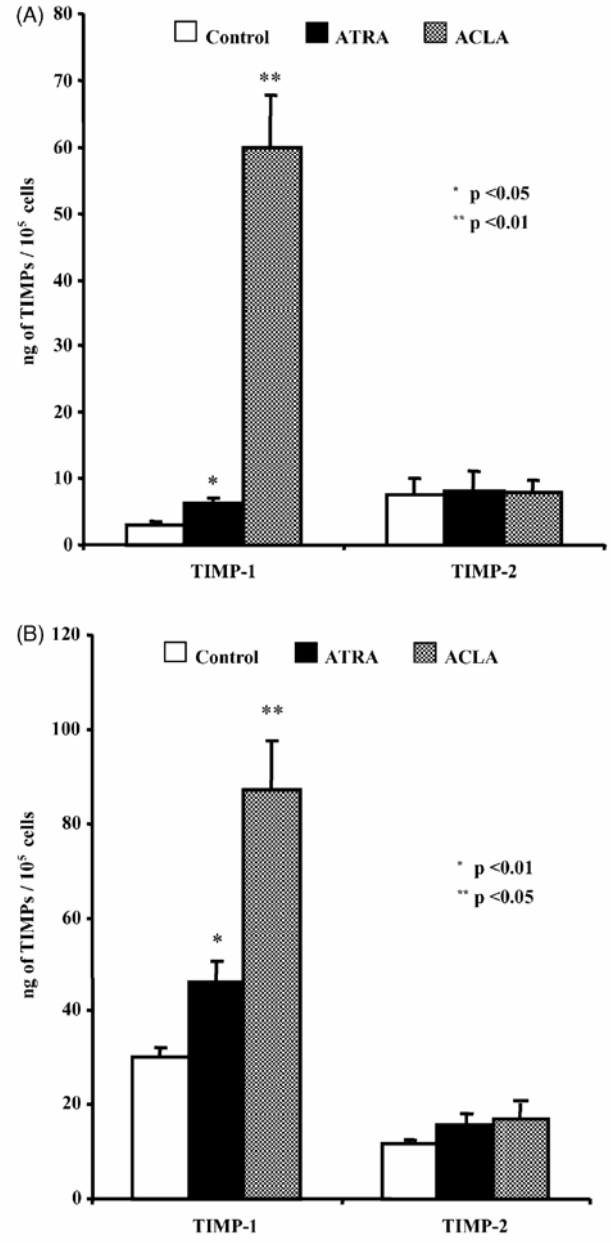

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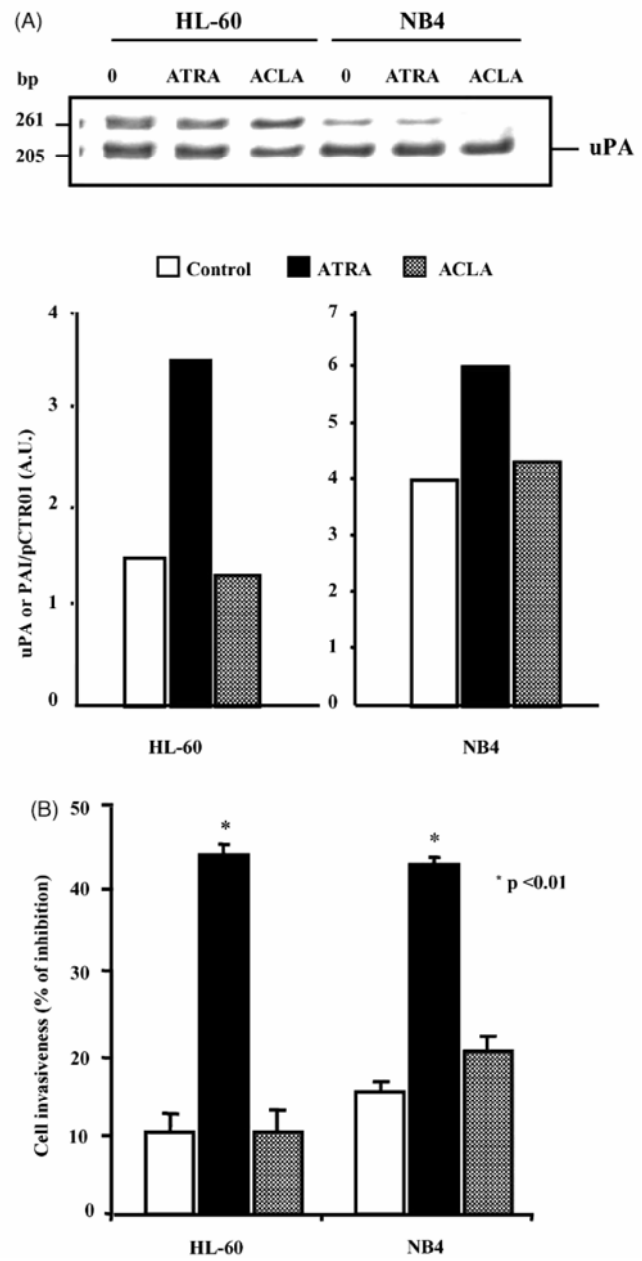
Fig. 4. *Expression of the serine protease uPA during induction of differentiation of leukemic HL-60 and NB4 cells.* (A) Competitive RT-PCR analysis of uPA was performed on total RNA. Endogenous uPA was detected at the size of 261 bp, pCTROl control bands for uPA was obtained at the expected sizes 205 bp uPA was expressed as a ratio to their specific internal control. The levels of mRNAs were normalized. (B) Effect of aprotinin, a serine protease inhibitor, on leukemic cell invasiveness. Results are expressed as the percentage of inhibition of cell invasion. Mean value of four experiments with triplicates performed in each, ± SD.



3.6. Expression of uPA by differentiated leukemic cells

As illustrated in Tables 3 and 4 and Figs. 1 and 2, ATRA-differentiated HL-60 and NB4 cells were more invasive although a minimal level of MMP-9 was produced. To clarify these apparently conflicting results, we then examined the mRNA production of the serine protease uPA which plays an important role in the plasmin-mediated MMP activation [22] and cell migration [23]. In both cell lines, uPA mRNA levels were increased with ATRA (Fig. 4A) and non-significantly modified in the presence of ACLA or ACLA*. Incubation with aprotinin, an inhibitor known to directly inhibit plasmin, during the invasion assay, inhibited by 42-44% the invasive properties of ATRA-differentiated NB4 and HL-60 cells. In contrast, aprotinin had no significant effect on ACLA or ACLA*-treated and control cells (Fig. 4B).

4. Discussion

In hematological disorders MMP expression has been especially analyzed in lymphoid malignancy [24] and to a lesser extent in myeloid ones [25]. In this respect, leukemic blasts of patients diagnosed with acute myelogenous leukemia have been shown to secrete gelatinases MMP-2 and MMP-9 [26] suggesting a possible role of these enzymes in leukemic cell dissemination.

In the present study, we analyzed the migrating and the invasive properties of human leukemic HL-60 and NB4 cells, their capacities to express MMPs, TIMPs and uPA in response to differentiating agents. In contrast to cytotoxic drugs, differentiating agents are used with the aim of inducing terminal differentiation and loss of self-renewal potential. The Vitamin A derivative ATRA induces differentiation of cells into mature granulocytes both *in vitro* with HL-60 (AML2) and NB4 (promyelocytic) cells, as well as in patients. It now constitutes the most successful therapy in acute promyelocytic leukemia [27]. Besides ATRA, anti-neoplastic agents, especially anthracyclines, have been shown to trigger differentiation of leukemic and tumor cells *in vitro* and *in vivo*, in animal models. In this study, we used the anthracycline ACLA as granulocytic inducer of HL-60 cells [28]. For the first time, we demonstrate that ACLA could also be used as an inducer of NB4 cells, which by contrast to HL-60 contains the typical translocation t (15; 17) associated with acute promyelocytic leukemia [29]. As demonstrated with other non-retinoid agents [30] such as hexamethylene bisacetamide, butyrates, ACLA* -induced differentiation of NB4 cells requires cell pre-exposure with a low concentration of ATRA.

The secretion of MMPs and TIMPs has already been investigated in leukemic cells [31] but, to our knowledge, no data is available concerning their expression during cell maturation. In addition, a limiting step when studying MMP secretion is that the presence of serum required for differentiation induction interferes with gelatin zymography and ELISA. This was overcome by using the UltraCULTURE® serum-free medium. Gelatinolytic activity of HL-60 and NB4 cells was compared to that of TPA-treated HT-1080 cells used as control for MMP expression. Leukemic cells are shown to mainly express MMP-9 (gelatinase B), which is consistent with earlier observations [32,33]. Compared to HL-60 cells, NB4 cells produced more MMP-9 as well as discrete levels of MMP-2 mRNAs. In addition, analysis of cellular extracts demonstrate that HL-60 and NB4 cells are able to activate pro-MMP-9, showing that they exhibit an autocrine activation mechanism of MMP-9, without the need for other cells as observed in solid tumors [34].

We also analyzed by RT-PCR the expression of MT-MMPs (1-3) which is known to activate *in vitro* the zymogen of MMP-2 [35,36]. MT2-MMP is predominantly expressed in NB4 cells, whereas MT3-MMP is absent from both cell lines tested. The absence of MT1-MMP mRNA in HL-60 cells confirms the previous report by Okada *et al.* [37]. None of the cell lines tested expressed the strome-lysins known for their broad substrate specificity for many extra-cellular matrix proteins.

As shown by a pioneer work of Janiak *et al.* [21] only a small percentage of leukemic cells exhibited an invasive potential through the matrigel barrier (1.0-3.0%) and migrating capacity (1.3-4.6%). These data confirm the heterogeneity within each leukemic cell population [38]. NB4 cells appeared to be more invasive than HL-60 cells, which could be related to a higher production of MMP-9 and/or MMP-2.

The differentiation of HL-60 and NB4 cells by ATRA, ACLA or ACLA* enhanced both cell migration and invasion. Most of the cells (>95%) that cross the matrigel filters were NBT positive (data not shown). In addition, kinetic studies showed that the number of migrating cells increase with the differentiated status of the cells (data not shown). These data strongly suggest that the increase percentage of migrating cells is not the result of a selection of immature migrating cells.

Their invasive behavior was significantly reduced by the synthetic MMP inhibitor BB-94, whereas cell migration through uncoated filters was not modulated, indicating that cell crossing to matrigel was MMP-dependent. These data suggest that differentiating agents such as ACLA and ATRA could enhance cell invasion both by influencing cell motility and by regulating cell invasion via a control of MMP activities. To address this latter possibility, we evaluated the effect of differentiating drugs on MMP secretion.

ACLA markedly stimulated expression of MMP-9 without any modulation of expression of uPA. These observations are consistent with the inhibition of cell invasion observed with the MMP inhibitor Batimastat, but not with the serine inhibitor aprotinin. In contrast, although ATRA-induced differentiation of HL-60 and NB4 cells strongly increased cell motility and cell invasion, it decreased MMP-9 production at gene and protein level. This apparent discrepancy between the low level of MMP-9 expression and the increased invasiveness of ATRA-differentiated cells could be explained by the involvement of an active uPA system [23]. Indeed, uPA mRNAs were stimulated by ATRA and aprotinin, a synthetic inhibitor of uPA, markedly inhibited differentiated cell invasiveness. These results indicate that a low level of active MMP-9 with presence of an active uPA system could be sufficient to enhance cell invasion. In addition, according to previous data [39,40], our results suggest that combinations of uPA and/or MMPs inhibitors with ATRA, would be of potential clinical benefit to prevent

ATRA syndrome, characterized by a tendency to bleeding and rapid mobilization of leukemic cells to various organs [41]. Dexamethasone is currently the only treatment for ATRA syndrome in APL patients. The mechanism of action of this corticosteroid is still unclear although *in vitro*, it does not affect migration and functional properties of NB4 and HL-60 cells [42,43]. It would be interesting to study its effect on MMPs and uPA expression in ATRA- or ACLA-treated leukemic cells.

Degradation of connective tissue is regulated, at least in part, by the balance between MMPs and their natural inhibitors TIMPs. Presence of TIMPs was demonstrated in both leukemic cell lines tested. High levels of TIMP-1 expression, which have been demonstrated to play a role in the maturation of hemato-lymphoblastoid cells [44] were detected in particular in NB4 cells, and increased during ACLA*-differentiation. In contrast, TIMP-2 expression did not significantly vary after differentiation induction. It is worth noting that TIMPs are multifunctional molecules, which can act as MMP inhibitors, and as modulators of cell proliferation and apoptosis [45].

In conclusion, these data demonstrate that HL-60 and NB4 cells secrete MMPs and uPA, which may be regulated specifically by differentiating agents such as antitumor drugs and can acquire certain functional capabilities of mature white blood cells. They also show that differentiating agents can act on the invasive capacities of the leukemic cells via different routes, in this case ATRA acts rather at the uPA level and ACLA directly on MMP-9 expression. Although, the precise molecular mechanisms of such activation are still unknown, they are specific to the differentiating effect of the drug; since in experimental conditions in which the drug only induced growth inhibition (absence of ATRA pre-treatment) no effect on MMP-9 expression was detected (data not shown). Finally these *in vitro* observations suggest that combinations of protease inhibitors with differentiating agents could constitute an efficient prophylaxis of the ATRA syndrome.

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