Involvement of reactive oxygen species in aclarubicin-induced differentiation and invasiveness of HL-60 leukemia cells

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Abstract - Aclarubicin (ACLA), which belongs to the anthracycline class of antineoplastic agents, has been demonstrated as a differentiating agent of human leukemia, including HL-60 cells. We report here on the incidence of ACLA-induced differentiation on matrix metalloproteinase (MMP) expression and cell invasiveness. The aim of this study was to investigate the involvement of reactive oxygen species (ROS) as mediators of ACLA-induced effects. By using a fluorescent probe, we showed that subtoxic (i.e. differentiating) concentration of ACLA generate reactive oxygen species in HL-60 cells. ACLA-differentiated cells exhibited an increased proMMP-9 secretion which has been observed by gelatin zymography and immunoassay. Antioxidants were able to inhibit ACLA-induced differentiation and proMMP-9 secretion. Furthermore, RT-PCR showed that ACLA increased MMP-9 and tissue inhibitor of MMP (TIMP-1) expression in a ROS-dependent manner. In addition, the migration and invasion capacities of HL-60 cells were enhanced by ACLA treatment, but only partially reversed by antioxidants. Altogether, these results evidenced ROS as messengers of ACLA-induced differentiation and MMP-9 expression.

INTRODUCTION

Aclarubicin (ACLA) belongs to the anthracycline class of antineoplastic agents widely used in conventional cancer chemotherapy of solid tumor and leukemia (1). While anthracyclines cytotoxic effects are commonly accepted to be due to DNA-intercalation and interaction with the nuclear enzymes DNA-topoisomerases (2), additional targets have been proposed such as alteration of the mitochondrial function (3), and production of free radicals (4, 5). The ability of anthracyclines to produce reactive oxygen species (ROS), in cells or tissues has been demonstrated with high concentration, as commonly used in cytotoxic chemotherapy (4, 5). Then, oxidative damages consecutive to anthracycline treatment were thought to explain the high cardiotoxicity of these drugs (6). Besides, when used at subtoxic concentration (nanomolar range) anthracycline, and especially ACLA, are potent differentiation inducers of solid tumor and leukemia (7, 8). It should be noticed that the subtoxic concentration of ACLA used in differentiation studies was 50-200 times lower than the plasmatic concentration found in patients undergoing classical chemotherapeutic treatment (9).

The human leukemia cell line HL-60 is derived from the peripheral blood of a 36 year old woman and represent a widely used model of acute myeloid leukemia type 2 (AML-2) (10). When exposed to differentiation inducers such as all-*trans*-retinoic acid (ATRA) or phorbol esters, HL-60 cells can be committed toward granulocytic or monocytic pathways, respectively (11, 12). Induction of granulocytic differentiation of HL-60 cells can also be achieved with subtoxic concentration of ACLA, whereas other anthracyclines were inefficient (13, 14). Cell differentiation was associated with adhesion and migration modifications as recently described in ATRA-treated leukemia cells (15, 16). Furthermore, several groups have shown that differentiation of HL-60 cells, when induced by either ATRA or phorbol ester, is accompanied with changes in their matrix metalloproteinases (MMPs) expression (17-19).

MMPs are a family of zinc-dependent endopeptidases that degrade all of the components of extracellular matrix (20, 21). MMP-2 and MMP-9 (gelatinases A and B), which are secreted as the latent forms proMMP-2 and proMMP-9, are the more frequently found in leukemia cells or cell lines (22). MMPs expression, secretion and activity are highly regulated, particularly by a family of highly specific inhibitor proteins called tissue inhibitors of metalloproteinases (TIMPs) (20, 23). Therefore, secretion of MMP-9 and/or MMP-2 was thought to be involved in the invasive properties of malignant cells (24), including leukemia (25, 26). Nevertheless, biological significance of MMPs and TIMPs expression is complex since TIMPs could also have growth-promoting effects and numerous MMPs as well as other matrix-degrading enzymes could be involved in the invasive process (20-24).

In the present work, we aimed first at evaluating the effect of ACLA on MMP and TIMPs expression, and invasiveness of HL-60 cells. The second aim was to investigate the role of ROS as mediators of ACLA-induced differentiation and increased MMP-9 expression. The results show that ROS are involved in ACLA-induced differentiation and MMP-9 overexpression, but only partially in ACLA-enhanced invasion.

MATERIALS AND METHODS

Chemicals

Matrigel®, a basement membrane preparation extracted from murine Engelbreth-Holm-Swarm tumors, was purchased from BD Biosciences (Le Pont de Claix, France). The C2938 fluorescent probe (6-carboxy-2',7'-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester)) was from Molecular Probes (Eugene, Oregon). ACLA and the antioxidants N-acetylcysteine (NAC) and pyrrolidine dithiocarbamate (PDTC) were purchased from Sigma

(L'Isle d'Abeau Chesnes, France), and were prepared as previously described (27), except NAC which was buffered to pH 7.4 with sodium hydroxide.

Cell culture

The human leukemia HL-60 cell line from the American Type Culture Collection (CCL-240) was maintained in RPMI 1640 medium plus glutamax (Life Technologies, Saint Quentin en Yvelines, France) supplemented with 15% heat-inactivated fetal calf serum (Life Technologies) under standard culture conditions. The presence of serum is required for differentiation induction in classical culture media but interferes with MMPs determination (gelatin zymography and immunoassays). Therefore, for all experiments, serum-free cultures of exponentially growing cells were performed in UltraCULTURE® medium (BioWhittaker, Emerainville, France) supplemented with 2 mM L-glutamine, which allow both differentiation and MMP determination (28). The cell number and viability were determined by the Trypan blue dye-exclusion method.

Differentiation assay

After 3 days of treatment with 25 nM ACLA, and in the presence or absence of antioxidants, the differentiation of HL-60 cells was assessed by nitroblue tetrazolium (NBT) dye reduction as previously described (11-13, 28). Cells containing blue-black formazan granules, indicative of the ability of HL-60 cells to generate superoxide anion during a phorbol ester-induced respiratory burst, were scored as differentiation positive. In addition, cytopsin of control or ACLA-treated cells were stained with Wright-Giemsa, and analysed by microscopy to allow the observation of granulocytic features (i.e. multilobular nucleus, prominent cellular indentation) as previously described (28).

Spectrofluorimetric determination of ROS

ROS were detected by using the carboxyfluoresceine derivative C2938 as described by Quillet-Mary *et al.* (29). Cells (1.10^6 /ml) were treated with 25 nM ACLA for 30 min to 6 h, washed, then incubated with 5 μ M C2938 in RPMI-1640 without phenol red (Sigma) for 30 min at 37°C. In some experiments PDTC was added 1 h before and during ACLA treatment at the final concentration of 1 μ M. After 3 washes in PBS, cells were resuspended in RPMI-1640 without phenol red and the fluorescence intensity was recorded between 505 and 600 nm with an excitation wavelength of 495 nm and a maximal emission wavelength of 520 nm.

Analysis of gene expression by RT-PCR

Total RNAs were extracted by using TRIZOL® reagent (Life Technologies) and 1 μ g was reverse transcribed by using the reverse transcription system from Promega (Charbonnières, France). One tenth of the resultant cDNAs were then amplified with recombinant Taq-DNA polymerase (Life Technologies) as followed: 94°C/90 sec; then *n* cycles consisting of 94°C/30 sec, *T_m*/60 sec and 72°C/60 sec, and a final elongation step of 72°C/10 min. The primer sequences, *T_m* values, and number of PCR cycles used were: MMP-9, sense GCGGAGATTGGGAACCAGCTGTA, antisense GACGCGCCTGTGTACACCCACA, *T_m* 68°C, *n*=30; TIMP-1, sense TCAGGCTATCTGGGACCGCAGGGA, antisense ACCATGGCCCCCTTTGAGCCCCTG, *T_m* 68°C, *n*=24 (29); TIMP-2, sense CGAGAAACTCCTGCTTGGGG, antisense CTCGGCAGTGTGTGGGGTC, *T_m* 68°C, *n*=27 (30); GAPDH, sense CTCTGCCCCCTCTGCTGATGC, antisense CCATCACGCCACAGTTTCCCG, *T_m* 60°C, *n*=23. The PCR products were analysed on a 1.4% agarose gel stained with ethidium bromide.

Gelatin zymography

Gelatinolytic activities in cell culture supernatants and cellular extracts were analyzed according to Heussen and Dowdle (32) with slight modifications. Cell culture supernatants were harvested after 3 days of culture in UltraCULTURE medium in the presence or absence of 25 nM ACLA and/or antioxidants. Cellular extracts were then obtained after lysis in 0.1 M Tris-HCl pH 8.1, 0.4% Triton X-100. An appropriate volume of cell culture supernatant or cellular extract corresponding to an equal amount of cells ($1-5 \times 10^3$ cells) was applied to 10% SDS-polyacrylamide gels containing 1 mg/mL gelatin. After electrophoresis, SDS was removed from the gel by two incubations in 2% Triton X-100 during 30 minutes. After overnight incubation at 37°C 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 minutes 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. The human fibrosarcoma HT-1080 cell line, which constitutively secrete proMMP-9 (92 kDa) and proMMP-2 (72 kDa) (33), was used as an internal control for MMP expression, as described elsewhere (28).

Invasion and migration assay

In vitro invasion was determined in the Matrigel-based assay as described previously by Janiack et al (34). Briefly, 13-mm polycarbonate filters of 8- μm pore size (Costar, Cambridge, MA) were coated with 50 μg Matrigel. The lower compartments of the transwell chambers were filled with serum-free UltraCULTURE® medium. Following indicated treatment, cells suspended in UltraCULTURE® medium were placed in the upper compartments (4×10^5 cells/chamber) and incubated for 6 hours at 37°C, in 5% CO_2 . After incubation, cells that had migrated through the Matrigel-coated filters were recovered from

the lower compartments and counted. The percentage of invasion was calculated by considering the number of cells in the initial cell suspension as 100%. 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.

Enzyme-linked immunosorbent assay (ELISA)

Following indicated treatment, the MMP-9, TIMP-1 and TIMP-2 proteins levels were quantified by ELISA (Biotrak™, Amersham-Bioscience, Orsay, France) in conditioned media from 10^5 cells, according to the manufacturer's instructions.

Statistics

Results from at least three independent experiments were given as mean $\pm$ SD, and one-way ANOVA with Student-Newman-Keuls post-hoc comparison was used for statistical significance with $p \leq 0.01$.

RESULTS

Inhibition of ACLA-induced differentiation by antioxidants

Previous studies have determined the optimal differentiating dose of ACLA in the 20 to 30 nM range (13, 28). Here, we have used a 25 nM concentration of ACLA to induce an average of 68% of cells being positive in the NBT-test after 3 days of treatment. The cytologic observation of ACLA-treated cells after Wright-Giemsa coloration confirmed the granulocytic differentiation of HL-60 cells (not shown). This differentiation was accompanied with a marked inhibition of cell growth (59%) but no cytotoxicity (less than 5% of dead

cells). As shown in Fig. 1, the ACLA-induced differentiation of HL-60 cells was strongly inhibited by antioxidants. Both NAC (5 mM) and PDTC (1 μ M), which have no significant effect on control cells, decreased the ACLA-induced differentiation to an average of 15% and 19% after 3 days of co-treatment, respectively. It is important to notice that NAC and PDTC increased the cell growth inhibition to an average of 70% and that higher concentration of these antioxidants became cytotoxic to HL-60 cells (not shown).

ROS production in ACLA-treated cells

In order to show ROS production in ACLA-treated HL-60 cells, we have used the carboxyfluoresceine derivative C2938, which is internalized in cells, then cleaved by intracellular esterase, and fluoresces in the presence of ROS (29). Cells were first treated with 25 nM ACLA for periods varying from 30 min to 6 h, washed, and then incubated at 37°C in the presence of 5 μ M C2938. The ROS production was determined by the measurement of the 520 nm fluorescence level in control and ACLA-treated cells at each time point. The results depicted in Fig. 2 shows a ROS production in ACLA-treated cells, which is maximal after 3 h of ACLA-treatment, and then declined to control level. In addition, the co-treatment of cells with ACLA and the antioxidant PDTC (1 μ M) inhibited the ACLA-induced ROS production (Fig. 2), indicating that fluorescence augmentation was related to ROS production. According to the literature, the antioxidant NAC has been avoided as it interfered with the fluorescence determination (35).

ACLA-induced MMP-9 expression was inhibited by antioxidants

Untreated HL-60 cells have been previously described to express mainly MMP-9 gelatinase activity (21, 36). Gelatin zymography analysis of cell culture supernatants indicated that proMMP-9 secretion was strongly increased by ACLA treatment and inhibited in the

presence of NAC and PDTC (Fig. 3A). Nevertheless, the inhibition of ACLA-induced proMMP-9 secretion was lower with PDTC than with NAC. This can be explained by the fact that PDTC alone increase the proMMP-9 secretion in control cells (Fig. 3A). The ROS-mediated increase of proMMP-9 in ACLA-treated cells, and the inhibitory effect of antioxidants, were confirmed by the quantification of proMMP-9 in cell culture supernatants by ELISA assay (Table I). Furthermore, the appearance of active MMP-9 could be observed in whole cell lysates from ACLA-treated cells, and was inhibited in the presence of antioxidant (Fig. 3B). At the mRNA level, MMP-9 gene expression was enhanced in the presence of ACLA, and NAC and PDTC acted as inhibitors of the ACLA transcriptional effect (Fig. 4). As observed with gelatin zymography, PDTC alone act as a weak stimulator of MMP-9 gene expression (Fig. 4).

Effect of ACLA and NAC on TIMP-1 and TIMP-2 expression and secretion

TIMPs are the physiological inhibitors of MMPs activity and their expression and/or secretion is thought to be coordinated with MMPs control. TIMP-1 and TIMP-2 levels were therefore determined by ELISA assay in cell culture supernatants. The results indicated that TIMP-1 secretion was increased in ACLA-treated cells with respect to control cells and strongly decreased in the presence of NAC together with ACLA (Table II). By contrast, TIMP-2 level was not significantly affected by ACLA-treatment (Table II). In addition, RT-PCR analysis showed that TIMP-1 mRNA expression was up regulated in ACLA-treated cells, whereas TIMP-2 mRNA level remained unchanged (Fig. 4). The antioxidants, NAC and PDTC, inhibited the effect of ACLA on TIMP-1 mRNA level, and were without effect on TIMP-2 mRNA level (Fig. 4).

Cell migration and invasiveness were increased by ACLA treatment

The above results suggest that ACLA is a modulator of the migration and/or invasion capacity of HL-60 cells. We have used transwell filters recovered, or not, by a Matrigel® layer to evaluate cell invasion or migration, respectively. As shown in Fig. 5A, an average of 3% of control HL-60 cells were able to cross the Matrigel® layer and the filter, this percentage was increased to 26% for ACLA-treated cells. The co-treatment with NAC slightly reduced the percentage of invasive cells to 21% ($p < 0.05$, Fig. 5A), suggesting the involvement of MMP-9, the expression of which was ROS-dependent. Furthermore, cell migration (i.e. in the absence of Matrigel®), which is independent of matrix degrading enzymes, was increased by ACLA treatment, but was unaffected in the presence of NAC (Fig. 5B).

DISCUSSION

Differentiation of leukemia cells has prompted increasing research interest as an alternative or support to conventional cytotoxic chemotherapy (37, 38). In addition to the well documented effect of ATRA (39, 40), some other molecules, including ACLA (7, 8) and butyric acid (38, 41), appear as potential differentiating agents. In particular, ACLA induces differentiation of K562 and HL-60 human leukemia cell lines (8, 12, 13). Nevertheless, the molecular mechanisms by which ACLA induces differentiation of leukemia cells are poorly understood. In the K562 cell line, a model of erythroid differentiation, we have previously demonstrated that ACLA act at the transcriptional level by increasing specific transcription factor synthesis and DNA-binding (8, 42). Furthermore our recent results suggested the involvement of oxidative stress in the ACLA-triggered differentiation of K562 leukemic cells (27). In these studies, subtoxic concentrations of ACLA were used to induce the erythroid differentiation of the human K562 cell line, and antioxidants were shown to inhibit the ACLA

effect (27). Here we have used the human HL-60 cell line to confirm the involvement of ROS production in the mechanism of action of ACLA in an other differentiation model. The clear inhibition of ACLA-induced HL-60 differentiation by antioxidant indicates that the ACLA-triggered ROS production is sufficient to engage most of the cells in the granulocytic differentiation pathway. However, the reason why only 75% of cell differentiate, and why the granulocytic pathway rather than the monocytic one is stimulated remain to be elucidated.

As a marker of both leukemia cell differentiation and invasive phenotype, we are interested in MMP expression in HL-60 cells. Previous reports have shown that HL-60 cells expressed and produced predominantly proMMP-9 (22, 36), which may contribute to leukemia dissemination (25, 26). We showed here that ACLA was able to increase the proMMP-9 secretion, and moreover induce the apparition of active MMP-9. Our present results also demonstrated that ACLA-treatment increase both migration (without Matrigel®) and invasion (with Matrigel®) capacities of HL-60 cells. Nevertheless, cell migration, which does not involve matrix degrading enzymes, was independent of ROS generation. By contrast, a part of the ACLA-triggered increase of invasion was mediated by ROS. This support the involvement of the ROS-dependent modulation of MMP-9 and TIMP-1 expression in the invasion process. However, other MMPs or matrix degrading enzymes would be stimulated by ACLA and involved in the ACLA-triggered enhancement of cell invasiveness. Such enzymes would be stimulated by ACLA through ROS-independent signal transduction pathway(s).

Results of RT-PCR analysis suggest that MMP-9 and TIMP-1 were up-regulated by ACLA at the transcriptional level and that ROS are mediators of this ACLA-effect. It is therefore tempting to hypothesize a role for redox-sensitive transcription factors such as NF- κ B and/or AP-1 which binding sites are present in the MMP-9 and TIMP-1 promoters (20-24). Stimulation of NF- κ B by anthracycline drugs (mainly daunorubicin) is quite

controversial (43-45). According to a previous report from Piret and Piette (45), we found in preliminary experiments that ACLA did not stimulate NF- κ B DNA-binding in HL-60 cells (DR and BC, unpublished data). By contrast, AP-1 DNA-binding was markedly increased between 1 and 3 hours after ACLA-treatment and then return to control level (DR and BC, unpublished data). This result is in agreement with the ROS-dependent stimulation of AP-1 DNA-binding by daunorubicin previously reported by Mansat-de Mas *et al.* (35). Nevertheless, further experiments are needed to identify the components of AP-1 complex which may be sensitive to ACLA, and how they are involved in MMP-9 and TIMP-1 gene expression.

To conclude, our results show for the first time that ACLA-induced differentiation and MMP-9 increased expression in HL-60 cells was mediated by the generation of ROS. In addition, our work show that ACLA increased HL-60 cell invasiveness, in part in a ROS dependent way.

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Table I. Determination of proMMP-9 level in HL-60 cell cultures.

Antioxidant	proMMP-9 (ng/10 ⁵ cellules) ^a	
treatment	Control	ACLA
None	0,86 ± 0,51 ^b	5,53 ± 1,76 ^c
NAC	0,51 ± 0,37	0,64 ± 0,45 ^d
PDTC	1,60 ± 0,62	2,62 ± 0,24 ^d

^a The level of proMMP-9 secretion was determined by ELISA in 3 days culture supernatants of control and ACLA-treated HL-60 cells in the presence or absence of antioxidant (NAC, 5 mM; PDTC, 1 μ M). ^b Data are the mean $\pm$ SD of three independent experiments, each done in triplicate. ^c Value significantly different from the control according to one-way ANOVA with Student-Newman-Keuls post-hoc comparison ($p < 0.01$). ^d Value significantly different from ACLA ($p < 0.01$).

Table II. Determination of TIMP-1 and TIMP-2 levels in HL-60 cell cultures.

Treatment	TIMP-1	TIMP-2
	(ng/10 ⁵ cellules) ^a	(ng/10 ⁵ cellules) ^a
Control	3.82 ± 0.96 ^b	2.33 ± 0.57
NAC	2.23 ± 0.88	2.25 ± 0.31
ACLA	15.54 ± 6.11 ^c	3.78 ± 2.08
ACLA + NAC	5.60 ± 0.87 ^d	4.46 ± 2.60

^a The levels of TIMP-1 and TIMP-2 was determined by ELISA in 3 days culture supernatants of control and ACLA-treated HL-60 cells in the presence or absence of NAC (5 mM). ^b Data are the mean ± SD of three independent experiments, each done in triplicate. ^c Value significantly different from the control according to one-way ANOVA with Student-Newman-Keuls post-hoc comparison (p<0.01). ^d Value significantly different from ACLA (p<0.01).

FIGURE LEGENDS

Fig. 1. Inhibition of ACLA-induced differentiation by antioxidants. HL-60 cells were treated, or not (Control), for 3 days with 25 nM ACLA in the presence, or absence, of 5 mM NAC or 1 μ M PDTC. The percentage of differentiation was assessed by the NBT test. Data are the mean $\pm$ SD of five independent experiments. Statistical significance was tested with one-way ANOVA with post-hoc Student-Newman-Keuls comparison; columns with different letters significantly differ from each other ($p < 0.01$).

Fig. 2. ROS production in ACLA-treated cells. HL-60 cells were treated, or not, for the indicated time with 25 nM ACLA in the presence, or absence, of 1 μ M PDTC before incubation with 5 μ M of C2938 probe for 30 minutes. For each time point, the fluorescence intensity was recorded by spectrofluorimetry (λ_{ex} , 495 nm; λ_{em} , 520 nm) and results were expressed as the relative fluorescence intensity (%) with respect to untreated cells. Results are the mean $\pm$ SD of three independent experiments. *Values significantly different from control according to one-way ANOVA with post-hoc Student-Newman-Keuls comparison ($p < 0.01$).

Fig. 3. Gelatin zymography analysis of (pro)MMP-9 expression. HL-60 cells were treated, or not (Control) for 3 days with 25 nM ACLA in the presence, or absence, of 5 mM NAC or 1 μ M PDTC. (A) Conditioned media were then harvested and proMMP-9 expression was analysed by gelatin zymography. (B) Whole cell lysates were prepared from an equal amount of cells and analyzed by gelatin zymography allowing the detection of active MMP-9 as indicated by the arrow. Result from a typical experiment representative of three.

Fig. 4. RT-PCR analysis of MMP-9 and TIMPs gene expression. HL-60 cells were treated, or not (Control) for 3 days with 25 nM ACLA in the presence, or absence, of 5 mM NAC or 1 μ M PDTC. Total mRNAs were then extracted and RT-PCR was performed as described in the Materials and Methods section. Result from a typical experiment representative of three.

Fig. 5. ACLA-mediated increase of HL-60 cells invasiveness and migration. HL-60 cells were treated, or not (Control) for 3 days with 25 nM ACLA in the presence, or absence, of 5 mM NAC. (A) The % of invasive cells was determined as the % of cells which have crossed the Matrigel®-coated filter after a 6h incubation period. (B) The % of migrating cells was determined as previously with uncoated filters. Results are the mean $\pm$ SD of three independent experiments, each done in triplicate. Statistical significance was tested with one-way ANOVA with post-hoc Student-Newman-Keuls comparison; columns with different letters significantly differ from each other ($p < 0.01$).

A

B