Evidence of the role of protein kinase C during aclacinomycin induction of erythroid differentiation in K562 cells

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Abstract At subtoxic concentrations, aclacinomycin is effective in controlling erythroid differentiation of K562, a human erythroleukemic cell line. To better understand early events implicated in this process, we have used bisindolylmaleimide (GF109203X), an inhibitor with a high selectivity for protein kinase C (PKC). Our data show that GF109203X inhibits aclacinomycin effects on K562, evidenced by a strong reduction of hemoglobinized cells and a marked decrease of mRNA rates of erythroid genes. To establish firmly PKC involvement, we also verified that aclacinomycin stimulates its rapid translocation, from the cytosolic to the membrane compartment. By Western blot analysis, we also show that after short induction times, PKCα was the most implicated.

Key words: Protein kinase C α; Erythroid differentiation; Aclacinomycin; GATA-1

1. Introduction

K562, a human erythroleukemic cell line, can undergo further differentiation in both megakaryocytic and erythroid lineages, depending on the stimulus [1]. We have previously shown that anthracycline antitumor drugs, at subtoxic concentrations, are effective in controlling erythroid differentiation, leading to the appearance of hemoglobinized cells [2]. Aclacinomycin (ACM) stimulates this process by an enhancement of the transcription of specific erythroid genes, inducing hemoglobin production [3,4]. Nevertheless, elements about early events implicated in the differentiation process, especially signal transduction pathways, remain to be elucidated.

Among multiple known signaling mechanisms, activation of protein kinases C (PKC), a family of phospholipid-dependent serine-threonine kinases, has been shown to play a key role in the processes of proliferation/differentiation of a wide variety of malignant cells, including K562 cell line [5–7]. However, little is known about erythroid differentiation induced by anthracyclines. These antitumor drugs, currently used in clinical oncology, are extremely active by provoking major damages of DNA. Now, they can also display their effect at the membrane level, acting on phospholipases which trigger specific cellular responses [8].

The aim of our investigation was to determine the role of PKC, if any, in the differentiating effect of ACM in K562 cells. We show that bisindolylmaleimide, GF109203X (GFX), a selective inhibitor of PKC [9], markedly decreased ACM induction of K562 erythroid differentiation. This was accompanied by a decrease of erythroid gene mRNAs, of GATA-1 protein, an erythroid-specific transcription factor, and by the inhibition of the activity of AP1, a transcription factor targeted by PKC. To establish firmly PKC involvement, we also verified that ACM stimulates rapid translocation of its activity, from the cytosolic to the membrane fraction. By Western blot analysis, we showed that after short time courses of induction, PKCα isoform was mainly implicated, as soon as 5 min of induction.

2. Materials and methods

2.1. Cell culture and reagents

Human K562 cells were grown in suspension culture in RPMI 1640 (Life Technologies, Cergy-Pontoise, France) supplemented with 10% heat-inactivated fetal calf serum (Life Technologies, Cergy-Pontoise, France) and with 2 mM L-glutamine. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Aclacinomycin was purchased from Sigma Chemical Co. (L'Isle d'Abeau Chesnes, France) and the PKC inhibitor GF109203X from Calbiochem (La Jolla, CA, USA). Monoclonal PKC and RACK1 antibodies were obtained from Transduction Laboratories (Lexington, KY, USA) and anti-GATA-1 monoclonal antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA). They were used according to the specifications of the manufacturers.

2.2. RNA analysis

RNA isolation was performed according to the method described by Chomczynski et al. [10]. 1 μg of total RNA was reverse-transcribed using the Superscript Preamplification System kit (Life Technologies, Cergy Pontoise, France). Then, 10 μl of cDNA was diluted to 50 μl in Taq polymerase buffer containing 0.2 mM of each dNTP, 1 μg of each primer, 0.1 μl [α-^{32}P]dCTP (10 μCi/μl, Amersham) and 1 U of Taq DNA polymerase (Gibco BRL). PCR conditions involved denaturation at 94°C for 90 s, annealing for 30 s at 60°C and extension at 72°C for 60 s, during 15 cycles for γ-globin, 20 cycles for NF-E2 and 25 cycles for PBGD. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. Aliquots of PCR were loaded on a 8% polyacrylamide gel. After drying, gels were subjected to autoradiography.

Primers for mRNA evaluation were selected using the program PCRBASE [11] and synthesized by Eurogentec (Seraing, Belgium). The sequences were: 5′-GGTCCTACTATCGCCTCCCTC-3′ (sense) and 5′-CCAGCCTCTGTCCCCTCCAGC-3′ (antisense) for PBGD; 5′-GGCAACCTGTCCTCTGCCTC-3′ (sense) and 5′-GCCAGGAAGCCTGCACCTCA-3′ (antisense) for γ-globin; 5′-ATTTGAGCCCCAAGCCCCAGC-3′ (sense) and 5′-CCAGCCTCTGTCCCCTCCAGC-3′ (antisense) for NF-E2. They generated PCR products of 256 bp for γ-globin, 217 bp for PBGD and 357 bp for NF-E2.

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Abbreviations: ACM, aclacinomycin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFX, GF109203X (bisindolylmaleimide); PBGD, porphobilinogen deaminase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; RACK, receptors for activated C kinase; TRE, TPA response element

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2.3. Western blotting

Proteins were separated on a 10% gel by SDS-PAGE and transferred onto nitrocellulose membrane (Amersham, Buckinghamshire, UK). The membrane was blocked by a 2 h incubation with 5% milk in 0.05% Tween 20-containing Tris-buffered saline. Immunodetections were performed by incubating the membrane with the specific antibodies and developed with appropriate IgG-horseradish peroxidase-conjugated secondary antibodies. Development was performed using chemiluminescent detection reagent (Pierce, Rockford, IL, USA).

2.4. Electrophoretic mobility shift assay

Nuclear proteins of 4×10^6 K562 cells were prepared according to Schreiber et al. [12] in the presence of protease inhibitors leupeptin, aprotinin, *o*-phenanthroline, benzamidine, phenylmethylsulfonyl fluoride. In vitro binding capacity to their consensus sequence and electrophoretic runs were performed as previously described by Montavani et al. [13]. The consensus nucleotide sequence used in these experiments was the TPA response element (TRE) [14]. It was purchased from Promega (Charbonnières, France).

2.5. Assays for PKC activity detection

PKC activity was evaluated in the particular and in the cytosolic fractions of K562 cells, according to the method described by Kikkawa et al. [15].

3. Results

Involvement of PKC during ACM-induced differentiation was first evaluated by using the specific inhibitor GF109203X. K562 cells were preincubated during 1 h with various concentrations of GFX, and then treated or not with ACM 20 nM. At day 3, differentiation rates were assessed by measuring hemoglobin accumulation, after benzidine staining. GFX inhibits effects of ACM in a dose-dependent manner and at 5 μM, the maximum non-toxic concentration, the differentiation rate decreased from 65% to 27%, suggesting a significant role of PKC during this process. In order to examine the effect of the inhibition of PKC on cell differentiation at the molecular level, we analyzed mRNA levels of various erythroid genes in presence or absence of GFX by RT-PCR.

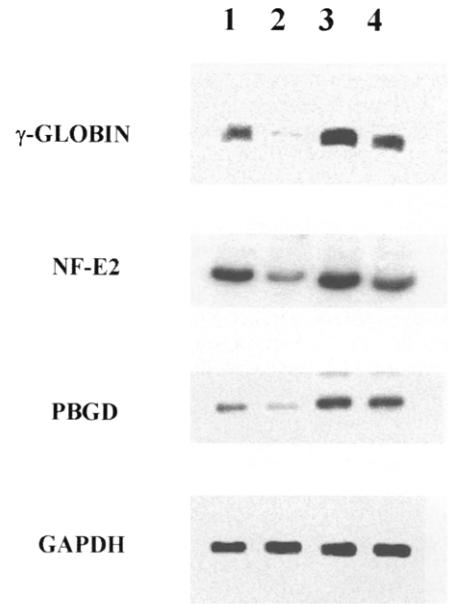

Fig. 1. GFX downregulation of erythroid mRNAs. RT-PCR analysis of γ-globin, PBGD and NF-E2 erythroid mRNAs in K562 cells induced or not to differentiate by ACM for 3 days. Untreated K562 cells (lane 1), GFX 5 μM (lane 2), ACM 20 nM (lane 3), GFX 5 μM+ACM 20 nM (lane 4).

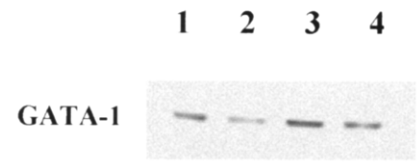

Fig. 2. GFX downregulation of the protein level of the erythroid transcription factor GATA-1. Western blot analysis of GATA-1 protein in K562 cells induced or not to differentiate by ACM for 3 days. Untreated K562 cells (lane 1), GFX 5 μM (lane 2), ACM 20 nM (lane 3), GFX 5 μM+ACM 20 nM (lane 4).

ACM treatment for 3 days led to a marked increase of mRNAs of γ-globin and porphobilinogen deaminase (PBGD), which participate in hemoglobin synthesis, and of NF-E2, an erythroid-specific transcription factor. In the presence of the PKC inhibitor, all these increases were suppressed, in spite of ACM treatment. Moreover, basal levels of these three erythroid markers were also reduced in uninduced cells only treated with GFX, whereas that of GAPDH internal control did not vary during this treatment (Fig. 1). Similar results were obtained at the protein level by examining GATA-1, another transcription factor that plays a central role in the regulation of erythroid differentiation [16]. Western blot analysis showed that GFX significantly abolished the GATA-1 protein increase observed after ACM stimulation (Fig. 2). Finally, since AP1 transcription factor is a target

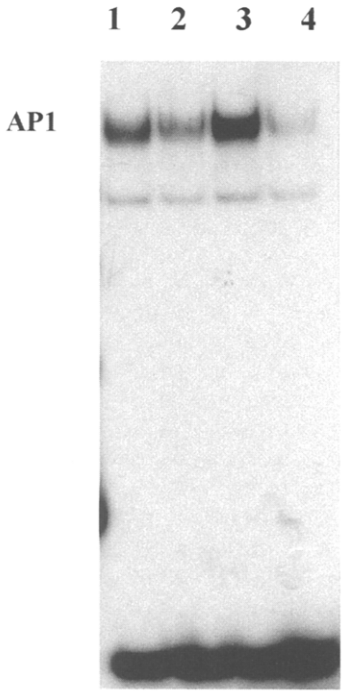

Fig. 3. GFX downregulation of AP1 activity. Nuclear extracts from K562 cells induced or not to differentiate by ACM for 3 days were incubated with labeled AP1 oligodeoxynucleotide and analyzed by gel mobility shift assay. Untreated K562 cells (lane 1), GFX 5 μM (lane 2), ACM 20 nM (lane 3), GFX 5 μM+ACM 20 nM (lane 4).

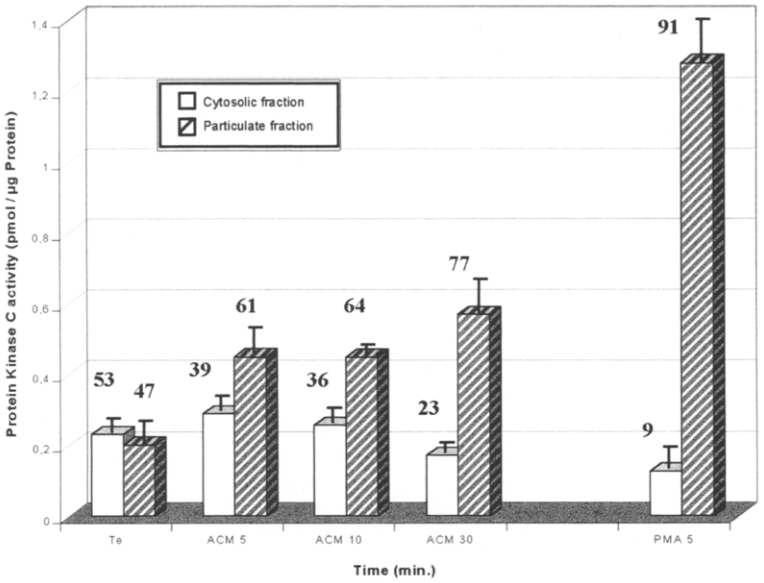

Fig. 4. ACM-induced rapid translocation and activation of PKC. PKC activity was measured into cytosolic (open bars) and particulate (hatched bars) fractions of K562 cells stimulated at various times with 20 nM ACM or 10^{-7} M PMA. Values in parentheses indicate the percentage of PKC activity into cytosolic and particulate fractions. Data represent the mean ± S.D. of triplicate determinations.

for PKC-regulated changes in gene expression [17], we measured its activity in erythroid cells by mobility shift assays, in the presence or not of ACM and GFX. Fig. 3 shows that stimulation of cells by ACM results in AP1 upregulation. This increase in AP1 activity was completely prevented by pretreatment with GFX.

These first results strongly suggest the possible role of PKC during K562 erythroid differentiation induced by ACM. This was verified by measuring the translocation of total PKC activity, from the cytosolic to the particulate (membrane) fraction after treatment by the anthracycline drug. Results in Fig. 4 show the rapid response of K562 cells to the ACM stimulus, since the translocation was detected within 5–30 min. At the basal state, PKC activity was 47% of the total activity (0.201 pmol/µg protein) in the particulate fraction. During the first 30 min of ACM treatment, a progressive increase of this PKC activity was found (61% after 5 min and 77% after 30 min). However, this increase in PKC activity was smaller than that observed with PMA, a reference activator of PKC.

In order to identify specific PKC isotypes implicated, we carried out immunoblot analysis of both particulate and cytosolic fractions after short time induction by ACM. Stripping and reprobing blots permitted us to analyze the same extracts with various specific antibodies. As shown in Fig. 5, PKC isotype analysis demonstrated that undifferentiated K562 cells express α, βI, βII and θ in both fractions. In our conditions ζ was only present in the cytosolic fraction and ε was no detectable (a whole rat brain lysate was used as a positive control, but no band appeared with K562 extracts). Induction with ACM led to a rapid activation of α isoform, which was translocated from the cytosolic to the particulate fraction within the first 30 min of treatment. No change was observed with the other isoforms tested. Finally, RACK1, a specific receptor for activated PKC [18], which is present at low levels in the cytosol, was strongly increased in the membrane fraction as soon as 5 min after ACM induction.

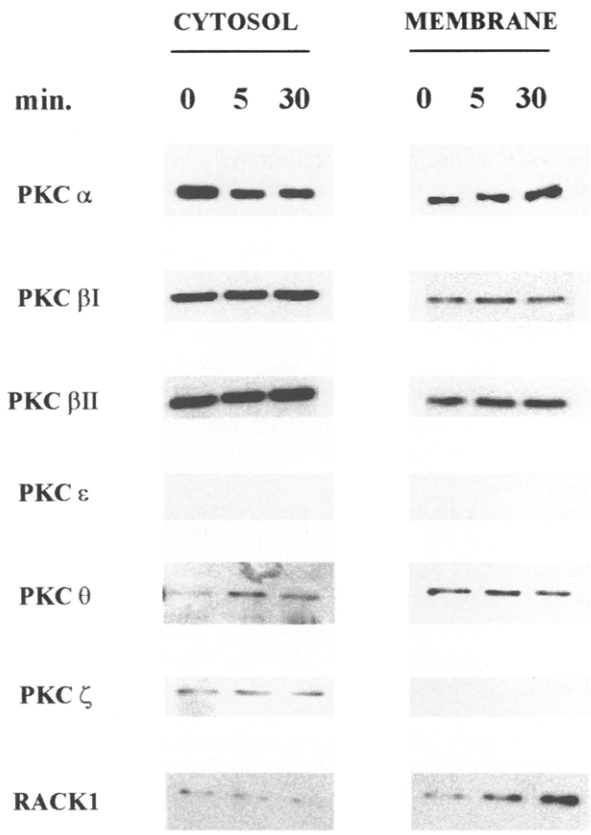

Fig. 5. Western blot analysis of PKC isoforms. Redistribution of PKC isoforms was evaluated after 5 and 30 min of treatment with 20 nM ACM. Cytosolic and membrane subfractions were prepared as described in Section 2. A representative of three separate experiments is reported.

4. Discussion

In this study we demonstrated for the first time that one mechanism by which the anthracycline antitumor drug ACM transduces the signal leading to erythroid differentiation was PKC-dependent. There was a strong reduction of erythroid differentiation by the GFX inhibitor, confirmed by the inhibition of various erythroid markers and of the binding of the ubiquitous transcription factor AP1, which is a well-known target for PKC [17]. Moreover, we observed a marked and rapid membrane translocation of the activity of total PKC after ACM treatment. This response was attributed to the α isotype and may implicate the anchoring protein RACK1, the level of which in the membrane fraction was also increased after ACM stimulation.

These data are in agreement with other studies that demonstrate a mechanism of action originating at the cell membrane level for anthracycline antitumor drugs. Indeed, we previously showed that covalent coupling of an anthracycline to a solid phase support, used to focus the drug action to the cell membrane, induces the appearance of differentiated cells without penetrating into the cells [19]. In addition, anthracycline antibiotics can be incorporated into phospholipid bilayer [20], where they interfere with membrane-associated enzymatic activities, leading to specific phospholipase C (PLC) activation [21]. In this case, the subsequent liberation of diacylglycerol could explain one of the mechanisms of action of ACM on PKC. According to this, the xanthogenate compound D609, a specific inhibitor of phosphatidylcholine-phospholipase C, was able to inhibit ACM-induced differentiation in a dose-dependent manner (unpublished results from our laboratory).

The role of PKC during the differentiation of leukemia cells is one of the most studied areas in hematology [22]. PKCα, whose role in normal and cancer cells is controversial, has been shown to contribute to cell differentiation in various systems, including the murine erythroleukemia Friend cell line. In this case, a temporal relationship between PKCα isoform localization to the nucleus and commitment to differentiation was established, indicating its important role in the signal transduction pathway to gene expression [23]. It should nevertheless be noticed that other isotypes can play a role during this process, depending on the differentiating agents used and with respect to the cell lines, on the differential modulation and subcellular distribution of PKC isotypes [24–27].

The next events leading to the appearance of the erythroid phenotype of K562 cells after anthracyclines treatment remain to be elucidated. Studies are ongoing to establish the relationship between transduction pathways, more particularly PKC, and activation of specific transcription factors like GATA-1, which when phosphorylated exhibits an increase in its binding activity to erythroid promoters [28].

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