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PLC-β2 Monitors the Drug-Induced Release of Differentiation Blockade in Tumoral Myeloid Precursors

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Abstract The differentiation therapy in treatment of acute promyelocytic leukemia (APL), based on the administration of all-trans retinoic acid (ATRA), is currently flanked with the use of As_2O_3, a safe and effective agent for patients showing a resistance to ATRA treatment. A synergy between ATRA and As_2O_3 was also reported in inducing granulocytic differentiation of APL-derived cells. We have demonstrated that phospholipase C-β2 (PLC-β2), highly expressed in neutrophils and nearly absent in tumoral promyelocytes, largely increases during ATRA treatment of APL-derived cells and strongly correlates with the responsiveness of APL patients to ATRA-based differentiating therapies. Here we report that, in APL-derived cells, low doses of As_2O_3 induce a slight increase of PLC-β2 together with a moderate maturation, and cooperate with ATRA to provoke a significant increase of PLC-β2 expression. Remarkably, the amounts of PLC-β2 draw a parallel with the differentiation levels reached by both ATRA-responsive and -resistant cells treated with ATRA/As_2O_3 combinations. PLC-β2 is not necessary for the progression of tumoral promyelocytes along the granulocytic lineage and is unable to overcome the differentiation block or to potentiate the agonist-induced maturation. On the other hand, since its expression closely correlates with the differentiation level reached by APL-derived cells induced to maturate by drugs presently employed in APL therapies, PLC-β2 represents indeed a specific marker to test the ability of differentiation agents to induce the release of the maturation blockade of tumoral myeloid precursors. J. Cell. Biochem. 98: 160–173, 2006.

Key words: acute promyelocytic leukemia (APL); phospholipase C-β2 (PLC-β2); ATRA; As_2O_3; NB4

A block of granulocytopoiesis characterizes acute promyelocytic leukemia (APL), a M3 subtype of acute myeloblastic leukemia, leading to an uncontrolled accumulation of abnormal heavily granulated promyelocytes in peripheral blood and bone marrow [Parmar and Tallman, 2003]. The vast majority of APL patients present a reciprocal and balanced translocation t(15;17), resulting in a fusion gene that encodes the chimerical protein PML/RARα, involved in the pathogenesis of the disease [Warrell et al., 1993; Melnick and Licht, 1999]. Even if the fusion protein impairs the normal functions of both native PML and RARα, APL cells show a strong sensibility to pharmacological concentrations of all-trans retinoic acid (ATRA), that induces both leukemic blasts and APL-derived cell lines to differentiate into mature granulocytes [Tallman et al., 1997]. Despite the great advances in the therapy of APL, approximately 20%–30% of patients relapse and thus require salvage therapy based on additional cycles of ATRA and chemotherapy, that are often toxic and that induce a high number of patients to develop resistance to retinoids [Soignet,

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2001; Miller and Waxman, 2002; Tallman et al., 2002; Avvisati and Tallman, 2003; Tallman, 2004]. In this context, the search of novel therapeutic approaches with efficacy for relapsed APL patients, or for patients manifesting a primary resistance to ATRA, is still the subject of a number of investigations.

On the basis of an extensive use in traditional Chinese medicine, As_2O_3 was introduced, about 10 years ago, in the treatment of patients with APL and it currently represents a safe and effective agent to induce complete remission in patients with relapsed and refractory APL [Miller and Waxman, 2002; Miller et al., 2002; Slack et al., 2002; Lazo et al., 2003; Evens et al., 2004]. In addition, recent studies performed on patients with newly diagnosed APL demonstrate that the use of ATRA/As_2O_3 combinations for remission and maintenance phases brings much better results than either of the two drugs used alone, in terms of quality of the complete remission and of the disease-free survival status [Shen et al., 2004; Wang et al., 2004].

In vitro studies on the APL-derived NB4 cell line indicate that relatively low concentrations of ATRA induce morphological changes and highly modulate the expression of differentiation-related cell surface antigens [Lanotte et al., 1991]. Also low doses of As_2O_3 induce a partial differentiation of APL-derived cells, while high concentrations inhibit cell growth mainly through apoptosis [Chen et al., 1997; Soignet et al., 1998]. Furthermore, a synergism between ATRA and As_2O_3 in inducing granulocytic differentiation has been observed in both wild-type NB4 and in NB4-derived cell lines resistant to As_2O_3 or ATRA [Gianni et al., 1998; Jing et al., 2001]. Even if ATRA, at variance with As_2O_3, acts through nuclear receptors, members of the transcription factors family, the granulocytic differentiation of APL cells induced by both ATRA and As_2O_3 involves the degradation of the leukemogenic protein PML/RARα [Zhu et al., 2001; Jing, 2004].

Several evidences provided by our and other groups indicate that ATRA-induced granulocytic differentiation of tumoral myeloid precursors involves enzymes related to the inositol lipid metabolism, like lipid-kinases and phospholipases [Zylber-Katz and Glazer, 1985; Iiri et al., 1995; Zauli et al., 1996; Bertagnolo et al., 1997, 1998, 1999, 2001, 2004; Marchisio et al., 1998]. We have recently reported that the β2 isoform of phospholipase C (PLC), highly expressed in neutrophils of peripheral blood, is nearly absent in APL-derived cells [Bertagnolo et al., 2002]. ATRA-induced granulocytic differentiation of both NB4 cells and blasts purified from patients with APL is coupled with a large increase of the expression of this protein. More remarkably, the level of PLC-β2 reached by APL blasts after ex-vivo treatment with ATRA strikingly correlates with the responsiveness of APL patients to ATRA-based therapies. In addition, we have demonstrated that a high expression level of PLC-β2 is a landmark of the normal granulocytic differentiation of primary $CD34^+$ hematopoietic progenitors, substantiating the contention that an adequate PLC-β2 expression has to be achieved, by normal and malignant precursors, for myeloid maturation [Bertagnolo et al., 2002].

A first aim of this work was to elucidate whether PLC-β2 is positively modulated during granulocytic maturation promoted by differentiating doses of As_2O_3 and of ATRA/As_2O_3 combinations, addressing the question of whether it may constitute a marker for monitoring the ability of different drugs to release the maturation blockade of tumoral myeloid precursors. We have also tried to ascertain if PLC-β2 directly promotes maturation and/or potentiates the agonist-induced differentiation of myeloid precursors, challenging the possibility that it represents a target for a gene-based therapy of APL.

MATERIALS AND METHODS

Cell Culture and Reagents

The NB4 APL-derived cell line was obtained from the "German Collection of Microorganisms and Cell Cultures" (Braunschweing, Germany). The NB4-Resistant (NB4-R) clone was kindly provided by Dr. Carlo Gambacorti-Passerini (Department of Experimental Oncology, Istituto Nazionale Tumori, Milan, Italy). Both cell lines were cultured in RPMI 1640 (Gibco Laboratories, Grand Islands, NY) supplemented with 10% fetal calf serum (Gibco) in a 94/6% air/CO_2 atmosphere. Cells were maintained at an optimal cell density between 5×10^5/ml and 1.5×10^6/ml.

ATRA and Arsenic Trioxide (As_2O_3) were purchased from Sigma Chemicals Co. (St. Louis, MO). ATRA stock solutions were obtained by dissolving the differentiating agent in absolute ethanol. Stock solutions of As_2O_3 were prepared

by dissolving the compound in a phosphate buffered saline (PBS, Cambres Bioscience, Verviers, Belgium) solution containing 100 μM NaOH.

Characterization of Granulocytic Differentiation

The degree of neutrophil-like differentiation of NB4 and NB4-R cells was evaluated by measuring the expression level of CD11b and CD11c myeloid surface markers by direct staining with phycoerythrin (PE)-conjugated anti-CD11b and anti-CD11c (Immunotech, Coulter Company, Marseille, France). Briefly, aliquots of 5×10^5 cells were incubated for 1 h at 4°C with 2 μl of Ab in 100 μl of PBS containing 2% FBS. Non-specific fluorescence was assessed by using an isotype-matched control, Mouse IgG1-PE (Immunotech) that shares certain structural characteristics with the monoclonal antibody, but is devoid of any relevant specificity with regard to the studied cell population. After staining, samples were analyzed by flow cytometry (FACScan, Becton-Dickinson, San José, CA) with Lysis II software (Becton-Dickinson). Data collected from 10,000 cells are presented as percentage of positive cells or as mean fluorescence intensity (MFI) values.

Immunochemical Analysis

For Western blot analysis, cells, after washing in cold PBS plus Na_3VO_4, were lysed in 1× Laemmli Buffer and boiled for 5 min. About 50 μg of protein was separated on 7.5% polyacrylamide denaturing gels and transferred to nitrocellulose membranes (Amersham Life Science, Little Chalfont, UK). The blots were then saturated for 1 h in tris buffered saline (TBS) containing 0.05% Tween-20 and 5% milk, and incubated overnight with a specific anti-PLC-β2 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). After incubation with an anti-rabbit IgG peroxidase-conjugated antibody (Sigma), the immunocomplexes were detected by chemiluminescence using the ECL system (Perkin-Elmer, Boston, MA), according to the manufacturer's instructions.

For the analysis of β-tubulin, the nitrocellulose membranes were saturated for 1 h in PBS with 3% BSA, then hybridized with the monoclonal specific anti-β-tubulin antibody (Sigma), incubated with an anti-mouse IgG peroxidase-conjugated antibody (Sigma) and revealed by chemioluminescence, as above described.

Densitometric analysis of autoradiograms were performed on ImageQuant TL (Amersham Bioscience).

Immunocytochemical Analysis

After two washes in PBS, cells were cytocentrifuged onto glass slides, fixed with freshly prepared 4% paraformaldehyde (10 min at room temperature), washed in PBS (5 min), and reacted with a polyclonal anti-PLC-β2 antibody (Santa Cruz) in NET gel (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, PH 7.4, 0.05% NP-40, 0.25% carragenin, 0.02% NaN_3), for 2 h at room temperature. Samples were then reacted with a secondary antibody (FITC-conjugated anti-rabbit IgG, Sigma) in NET gel for 45 min at room temperature. After two washes with NET gel and PBS, respectively, samples were incubated (from 30 s to 5 min) with 0.5 μg/ml DAPI, then washed in PBS, dried with ethanol, mounted in glycerol containing 1,4-diazabicyclo [2.2.2] octane to retard fading and analyzed with a fluorescence microscope (Axiophot 100, Carl Zeiss, Oberkochen, Germany), as previously reported [Bertagnolo et al., 1997].

RNA Interference Assay

Specific siRNAs was designed by selecting four target sequences in the coding region of PLC-β2 mRNA (Accession Number NM 004573), which fulfilled the specific sequence requirements as follows: AA (N_{19})dTdT (N is any nucleotide); 21-nt sense and 21-nt antisense strand; ~50% G/C content; and a symmetric 2-desoxythymidine 3′ overhang. Sense and antisense sequences were chemically synthesized by Qiagen (Qiagen S.p.A., Milan, Italy). The lyophilized siRNA was dissolved in sterile suspension buffer (100 mM potassium acetate, 30 mM HEPES-KOH, 2 mM magnesium acetate, pH 7.4) to obtain a 20 μM solution, and then nucleotides were heated to 90°C for 1 min followed by 1 h at 37°C. As a control, non-silencing fluorescein-labeled duplex siRNA, purchased from Qiagen, was used.

Twenty-four hours before transfection, cells were cultured at a density of 5×10^5 cells/ml of medium. Cells were transfected in contemporary with all siRNA duplexes using an electroporation procedure by means of an Electro Square Porator ECM 830 (Genetronics, Inc., San Diego, CA).

Briefly, 8 μg of siRNA in suspension buffer were mixed with 2×10^6 cells in 100 μl of RPMI plus 20% FBS. Cells were electroporated with a pulse of 250 V for 30 ms and, following electroporation, cells were allowed to recover in 600 μl of RPMI culture medium with 20% FBS.

After 5 h, cells transfected with fluorescein-labeled siRNA were subjected to cytofluorimetric analysis to determine transfection efficiency, that is about 60%, and cells transfected with silencing siRNAs were treated with agonists. After 48 and 72 h of culture, respectively, transfected cells were subjected to Western Blot analysis of PLC-β2 expression and, after 72 h of differentiating treatments, to cytofluorimetric analysis of CD11b and CD11c surface antigens.

PLC-β2 Overexpression

NB4 and NB4-R cell lines were transfected with plasmids expressing EGFP and EGFP-tagged PLC-β2, respectively. Briefly, 5×10^6 cells were mixed with 10 μg of plasmid DNA in TE solution (10 mM Tris-HCl and 1mM EDTA), in 400 μl of RPMI plus 20% FBS, then electroporated with a pulse of 250 V for 30 ms. Following electroporation, cells were allowed to recover in 8 ml of RPMI culture medium with 20% FBS. After 5 h of incubation, cells were centrifuged, resuspended in RPMI plus 20% FBS at a density of 5×10^5 cells/ml, and cultured for 72 h in control conditions or in the presence of the different agonists.

DNA constructs containing EGFP and EGFP-tagged PLC-β2 full-length were obtained subcloning the specific fragments into expression vectors (Amersham Pharmacia Biotech, Inc, Uppsala, Sweden) in the correct reading frame, amplifying plasmid DNA in competent bacteria and extracting DNA by means of a specific purification kit (Qiagen Plasmid Maxi Kit).

Statistical Analysis

The results were expressed as mean ± standard deviations of three or more experiments performed in duplicate. Statistical analysis was performed using the two-tailed Student's *t*-test for unpaired data.

RESULTS

Differentiating Doses of As_2O_3 Induce PLC-β2 Expression

In a first group of experiments, the APL-derived cell line NB4 was cultured for 96 h in the presence of As_2O_3 concentrations ranging from 0.1 to 1 μM. By daily monitoring cell growth, mortality, and differentiation level, we found that the higher concentration (1 μM) progressively reduced cell growth (Fig. 1A) and induced a significant increase of cell mortality (Fig. 1B) together with a slight progression of maturation, as measured by immunophenotypical analysis of the surface antigens CD11b and CD11c (Fig. 1C).

Lower concentrations of the agonist (in the 0.1–0.5 μM range) did not significantly affect the growing profile (Fig. 1A) and induced a relatively low cell mortality, without significant differences due to the doses or to the duration of treatments (Fig. 1B). The induced differentiation levels are reported in Figure 1C, which shows that As_2O_3 did not significantly modulate CD11b expression, whilst CD11c was upregulated, in terms of number of positive cells and fluorescence intensity, particularly when cells were treated with 0.5 μM As_2O_3.

The immunochemical analysis (Fig. 1D) revealed a weak but significant increase of the basal level of PLC-β2, particularly when cells were treated with 0.5 μM As_2O_3, in parallel with the acquisition of the highest differentiation level (Fig. 1C).

As_2O_3 Cooperates with ATRA to Induce Both Differentiation and PLC-β2 Expression

Since accumulating evidences [Gianni et al., 1998; Jing et al., 2001] suggest the existence of a synergism between ATRA and As_2O_3 in promoting differentiation of tumoral promyelocytes, NB4 cells were induced to differentiate in the presence of combinations of 1 μM ATRA, a concentration usually employed to differentiate tumoral myeloid cells, and variable amounts of As_2O_3 in the 0.1–0.5 μM range. As shown in Figure 2A, the cytofluorimetric analysis of CD11b and CD11c expression demonstrated that only at the concentration of 0.5 μM, As_2O_3 induced a significant upregulation of both antigens, compared to the levels obtained with the sole ATRA treatment.

The immunochemical analysis demonstrated that the amount of PLC-β2, already elevated when cells are grown with ATRA, showed a further slight increase when NB4 cells were treated simultaneously with ATRA and differentiating concentrations of As_2O_3 (Fig. 2B).

Since As_2O_3 exerts, alone or in combination with ATRA, the best therapeutic effects in

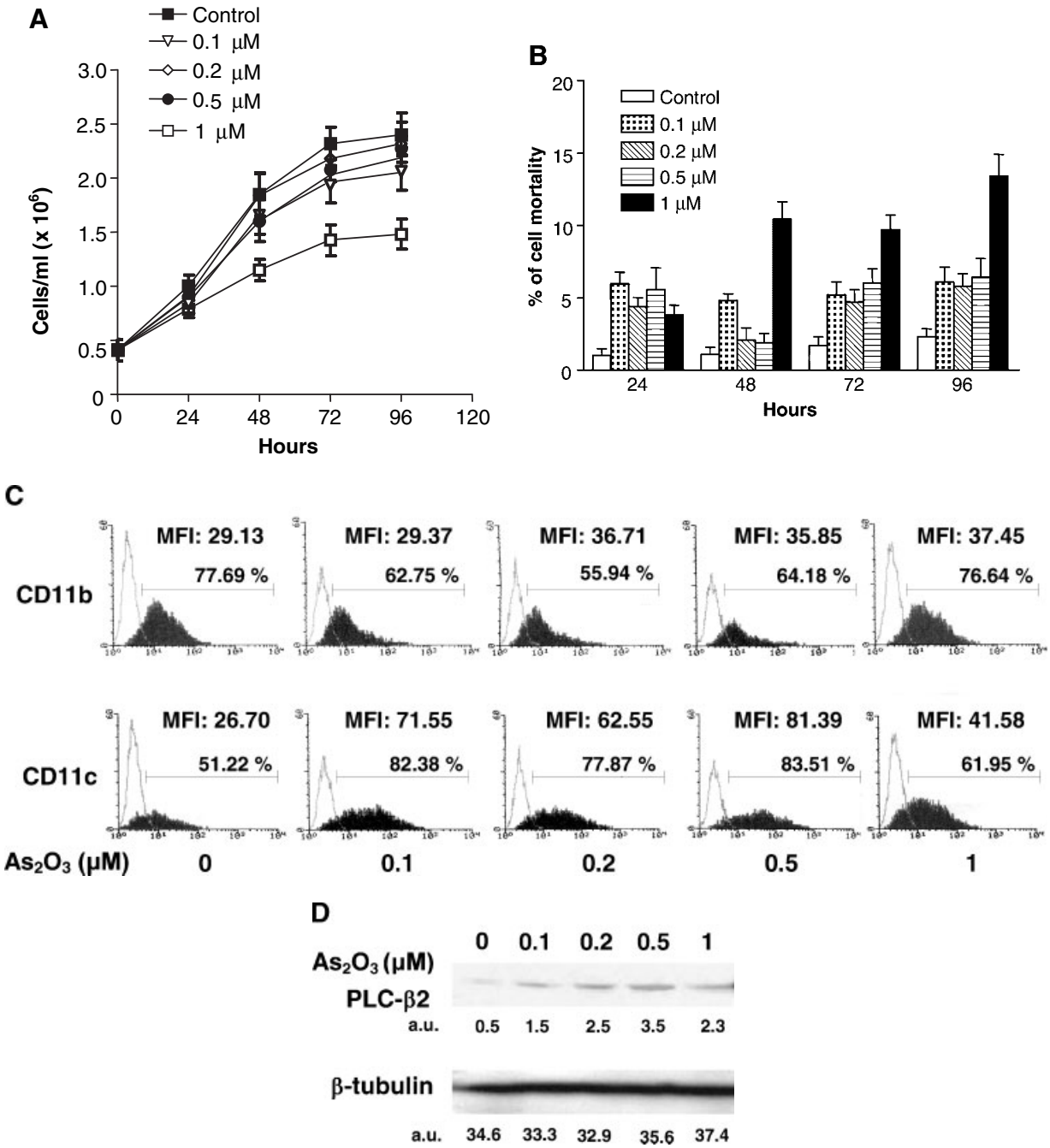

Fig. 1. NB4 cells were cultured in the presence of different concentrations of As_2O_3 (from 0.1 to 1 μM) and cell growth (**A**) and viability (**B**) were evaluated at various times of treatment (from 24 to 96 h). In the same experimental conditions, cytofluorimetric analysis of the indicated surface antigens was performed (**C**). The date indicate the percentage of positive cells and the mean fluorescence intensity (MFI). After 96 h of differentiating treatments, cell lysates were subjected to immunochemical analysis with the indicated antibodies (**D**). The values obtained after densitometric analysis of the autoradiograms are indicated as arbitrary units (a.u.). The data are representative of three separate experiments performed in duplicate.

refractory APL patients or patients who develop resistance to the ATRA-based therapy, we investigated the differentiating effects of As_2O_3 and of combination of ATRA and As_2O_3 on NB4-R, an NB4-derived cell line only moderately responsive to ATRA. In this group of experiments, we used the 0.5 μM As_2O_3 concentration, shown here to induce a partial

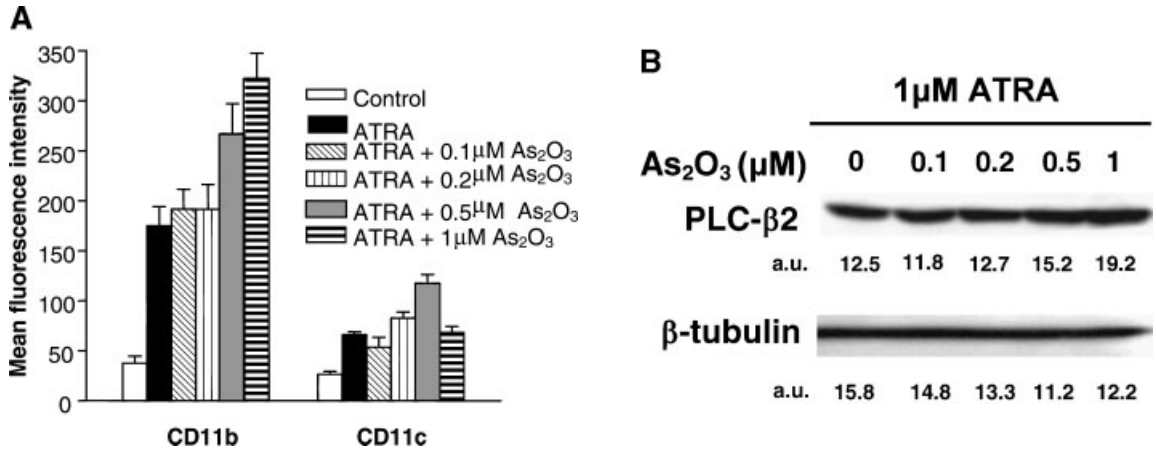

Fig. 2. NB4 cells, cultured in control conditions, in the presence of 1 μM ATRA and with different concentrations of As_2O_3 (from 0.1 to 1 μM) in combination with 1 μM ATRA for 96 h, were subjected to cytofluorimetric analysis of surface antigens expression (**A**) and to immunochemical analysis with the indicated antibodies (**B**). The values obtained after densitometric analysis of the autoradiograms are reported as a.u. The data are representative of three separate experiments performed in duplicate.

differentiation in NB4 cells without drastically modifying cell growth and viability (Fig. 1A,B,C).

NB4-R cells were hence treated for 4 days with either 1 μM ATRA or 0.5 μM As_2O_3, and with the two agonists in combination, and subjected to cytofluorimetric analysis of surface markers. Each of the two single agonists induces a similar, weak but significant, increase of both CD11b and CD11c levels (Fig. 3A). The amount of PLC-β2, nearly undetectable in control conditions, weakly increased, in a time-dependent manner, when cells were treated with As_2O_3 (Fig. 3B). On the other hand, the administration of ATRA upregulated the expression of PLC-β2 after 48 h and independently of the duration of treatment. Of note, after 96 h of treatment, ATRA and As_2O_3 induced a similar level of PLC-β2, consistent with a comparable expression of the differentiation markers (Fig. 3A). The combined administration of ATRA and As_2O_3 induced higher differentiation levels, as deduced by the expression of both surface antigens (Fig. 3A), and a PLC-β2 expression significantly larger than that observed after single differentiative treatment with each of the two agents (Fig. 3B).

The immunocytochemical analysis of differentiating NB4-R cells confirmed the slight increase of PLC-β2 expression due to the independent administration of As_2O_3 or ATRA, and the relevance of the amount of this protein induced by the combination of the two agonists (Fig. 3C). The nuclear staining with DAPI reveals the maturation-related changes of nuclear morphology, particularly evident when cells were cultured in the presence of the ATRA/As_2O_3 combination able to induce the higher differentiation level (i.e., ATRA + As_2O_3, Fig. 3A).

PLC-β2 Levels Closely Correlate with Phenotypical Differentiation

The existence of a synergism between ATRA and As_2O_3 in inducing the maturation of both ATRA-responsive and -resistant cell lines suggests that elevated differentiation levels could be obtained also in the presence of minimal concentrations of the two agonists.

As shown in Figure 4A, when NB4 cells were cultured in the presence of ATRA at concentrations below 1 μM, the simultaneous administration of As_2O_3 induced higher differentiation levels, and the combination of 0.1 μM ATRA with 0.5 μM As_2O_3 was able to induce the better granulocyte-like phenotype. Since, in NB4 cells, ATRA induces an elevated expression of PLC-β2 also when employed at less than 1 μM, only a slight, although significant, further increase of the amount of this protein was found in the presence of the different ATRA and As_2O_3 combinations (Fig. 4B).

The same differentiative treatments were performed on the ATRA-resistant NB4-R cells in which the cytofluorimetric analysis of the surface antigen CD11b demonstrated that the level of granulocytic differentiation reached by these cells increased in a way As_2O_3- and ATRA-dose dependent (Fig. 4C). In particular, when

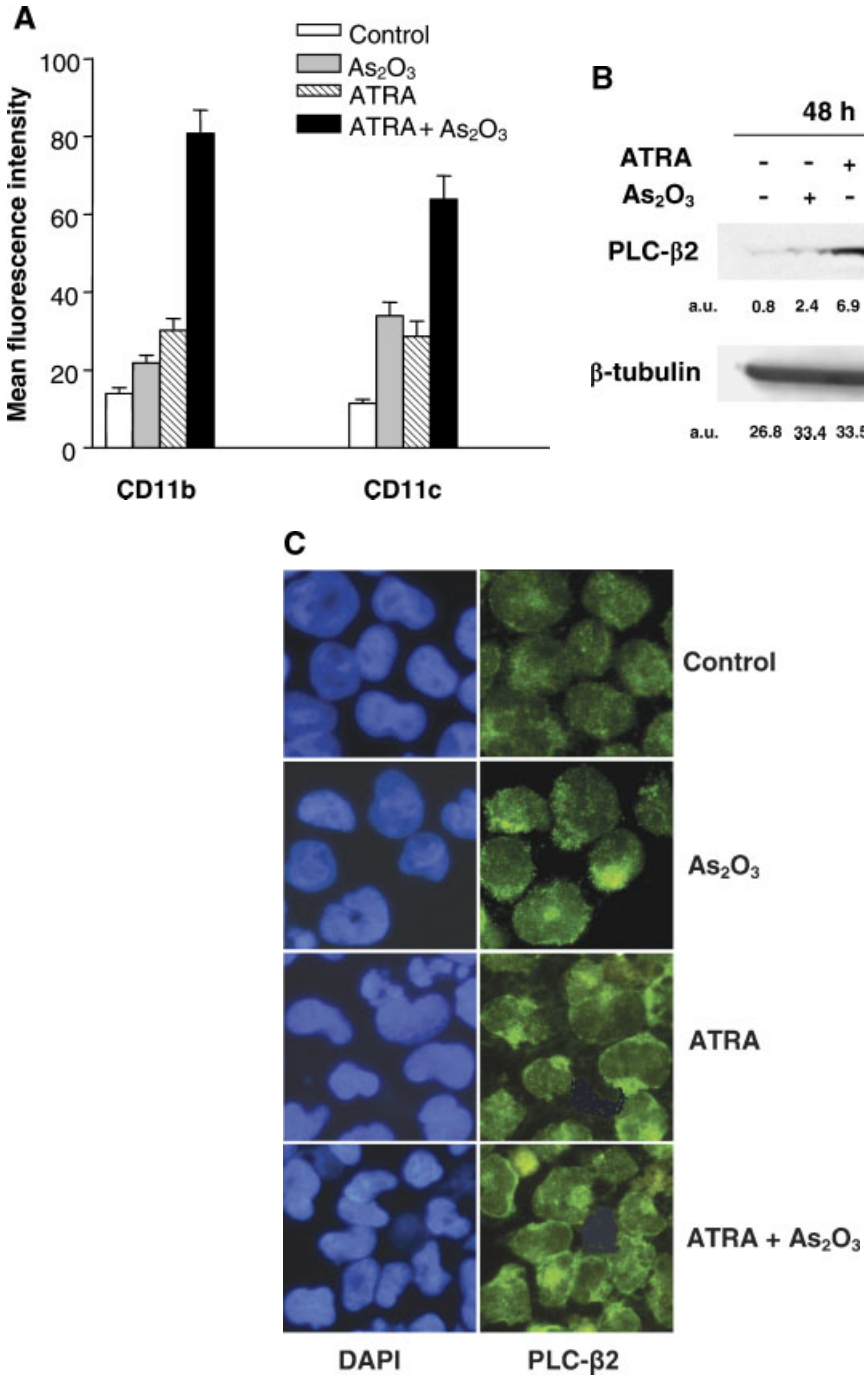

Fig. 3. NB4-R cells, cultured for 96 h in control conditions with 0.5 μM As_2O_3 and/or with 1 μM ATRA, were incubated with fluorescent antibodies against the myeloid surface markers and subjected to cytofluorimetric analysis (**A**). The same cells, after 48 and 96 h of treatment, were subjected to Western blot analysis with the indicated antibodies (**B**). The values obtained after densitometric analysis of the autoradiograms are indicated as a.u. The data are representative of three separate experiments, performed in duplicate. After 96 h of differentiating treatments, NB4-R cells were subjected to immunocytochemical analysis with the anti-PLC-β2 antibody (**C**). The nuclear morphology was detected by specific staining of DNA with DAPI. The pictures are representative of a group of three separate experiments performed in duplicate. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

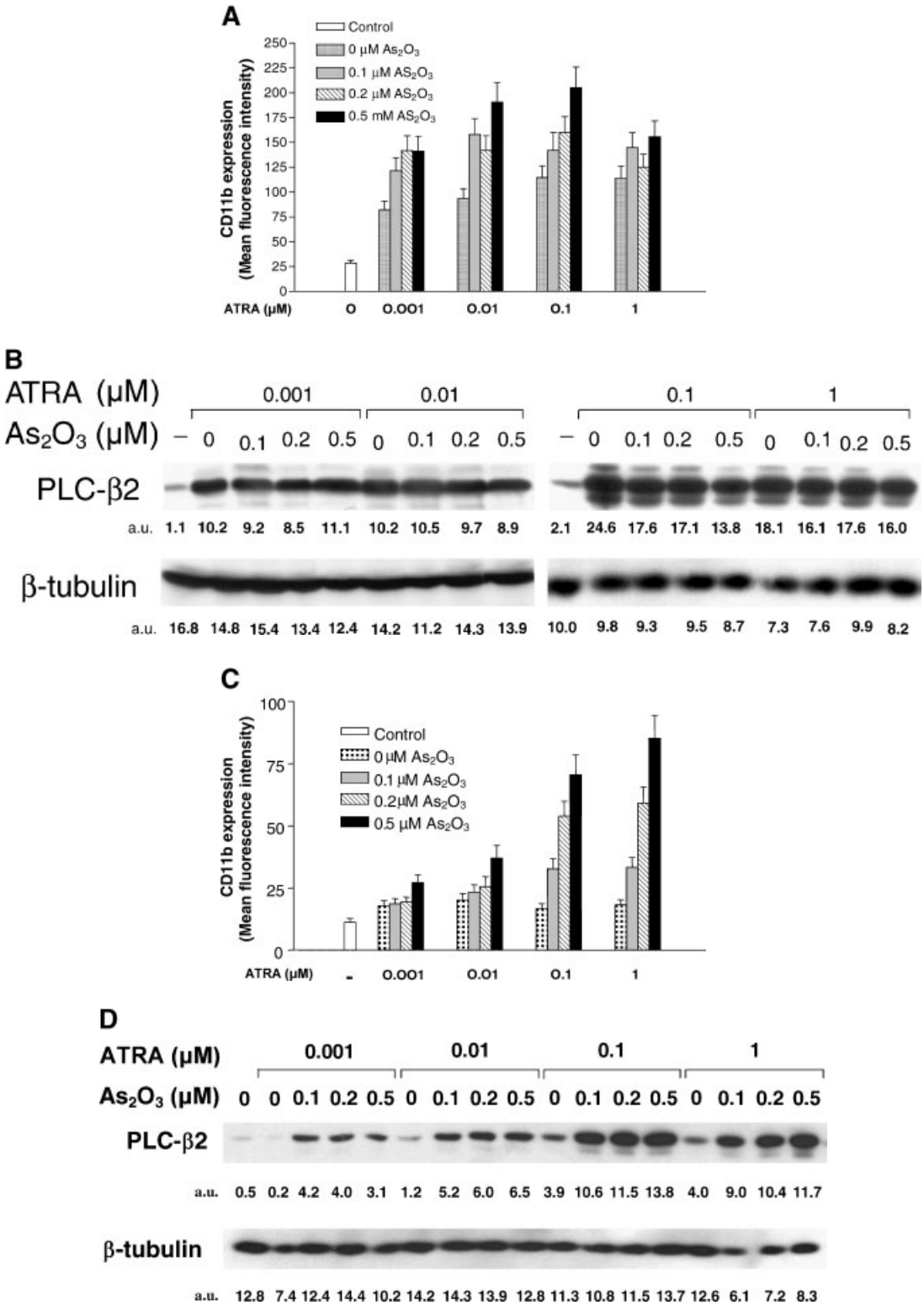

Fig. 4. NB4 (**A**, **B**) and NB4-R (**C**, **D**) cells were cultured for 96 h in control conditions and in the presence of combinations of different concentrations of As_2O_3 (from 0.1 to 0.5 μM) and ATRA (from 0.001 to 1 μM), then incubated with a fluorescent antibody against CD11b and subjected to cytofluorimetric analysis (A, C). The same cells were analyzed by Western blot with the indicated antibodies (B, D). The values obtained after densitometric analysis of the autoradiograms are indicated as a.u. The data are representative of three separate experiments performed in duplicate.

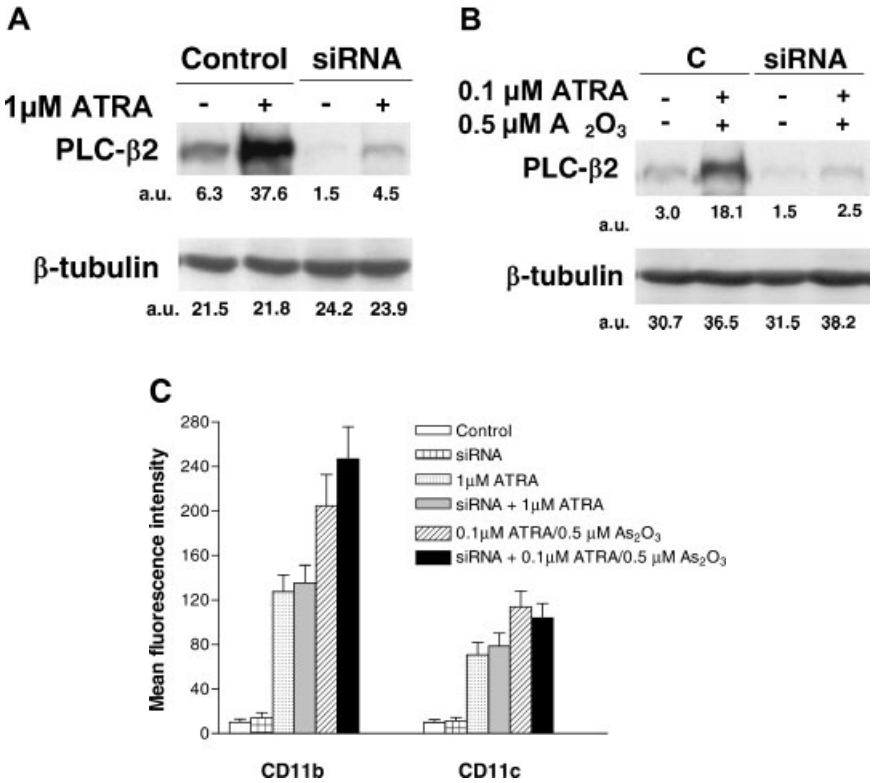

Fig. 5. NB4 cell were subjected to RNA interference assay (siRNA) for downmodulate PLC-β2 expression, and cultured in the presence of 1 μM ATRA (**A**) or in the simultaneous presence of 0.1 μM ATRA and 0.5 μM As_2O_3 **(B)**, then subjected toWestern blot analysis with the indicated antibodies to evaluate PLC-β2 expression. The values obtained after densitometric analysis of the autoradiograms were indicated as a.u. After 72 h of differentiating treatments, cells are subjected to cytofluorimetric analysis to estimate their differentiation level (**C**). The data are representative of three separate experiments performed in duplicate.

low concentrations of ATRA (0.001 and 0.01 μM) were added, only the concomitant presence of 0.5 μM As_2O_3 induced a slight but significant increase of the surface marker. In the presence of 0.1 and 1 μM ATRA, the maturation levels were progressively higher along with the increase of As_2O_3 concentration, being the combination 0.5 μM As_2O_3/1 μM ATRA the most effective in inducing the differentiation of this cell line (Fig. 4C).

The immunochemical analysis (Fig. 4D) demonstrated that the amount of PLC-β2 increases in parallel with the differentiation levels deduced from the expression of the differentiation marker (as reported in Fig. 4C), under all the explored combinations of ATRA and As_2O_3.

PLC-β2 is not Relevant for the Release of the Differentiation Block of Tumoral Myeloid Precursors

On the basis of the above reported findings showing that an increased expression of PLC-β2 takes place during ATRA- and/or As_2O_3-dependent maturation of tumoral myeloid precursors, we next addressed the question of whether PLC-β2 plays any role in the release of the differentiation block. With this aim, we firstly counteracted the agonist-induced increase of PLC-β2 by downmodulating its expression during the differentiative treatments. As reported in Figure 5, the reduced expression of PLC-β2 during treatment of NB4 cells with both ATRA (Fig. 5A) and the most effective ATRA/As_2O_3 combination (Fig. 5B), did not result in any significant change of the granulocytic differentiation levels (Fig. 5C). Similarly, when PLC-β2 was reduced during NB4-R treatment with the most effective ATRA/As_2O_3 combination (Fig. 6A), no modifications of the reached differentiation levels were observed (Fig. 6B).

A further set of experiments was aimed to assess whether a forced expression of PLC-β2 is capable to promote spontaneous differentiation or to potentiate the agonist-induced maturation

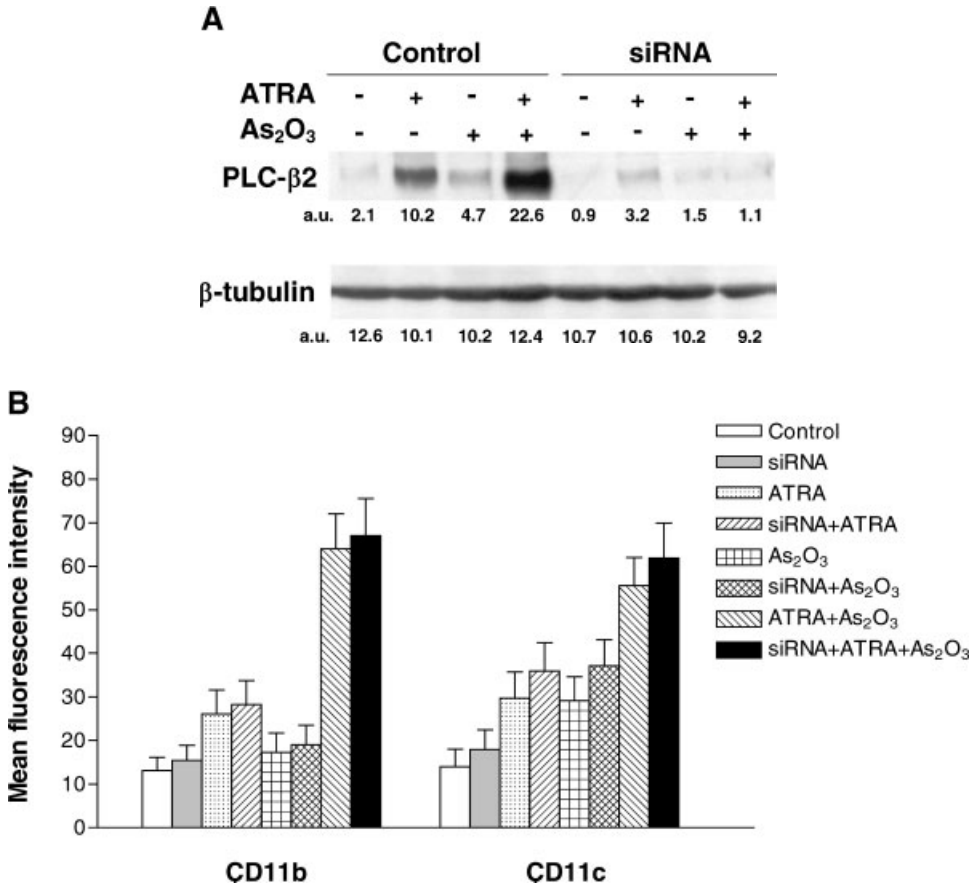

Fig. 6. NB4-R cell were subjected to RNA interference assay (siRNA), for downmodulate PLC-β2 expression, and cultured in the presence of 1 μM ATRA and/or 0.5 μM As_2O_3, then subjected toWestern blot analysis with the indicated antibodies (**A**). The values obtained after densitometric analysis of the autoradiograms are indicated as a.u. After 72 h of differentiating tretaments, cells were subjected to cytofluorimetric analysis of CD11b and CD11c expression (**B**). The data are representative of three separate experiments performed in duplicate.

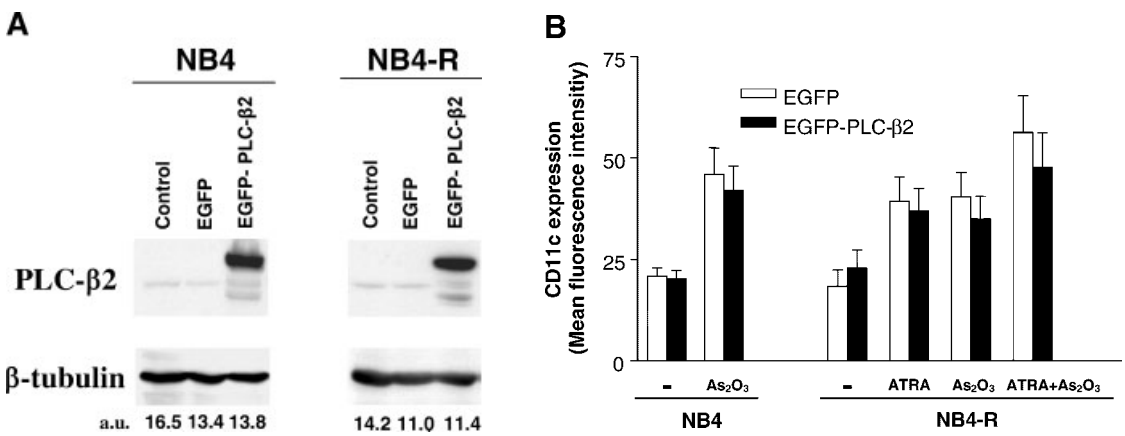

Fig. 7. NB4 and NB4-R cells were transiently transfected with an EGFP-tagged construct containing the full length cDNA of PLC-β2 (EGFP-PLC-β2) and subjected to immunochemical analysis with the indicated antibodies (**A**). Transfected cells were then cultured in control conditions or in the presence of 0.5 μM As_2O_3 and of 1 μM ATRA/0.5 μM As_2O_3, then subjected to cytofluorimetric analysis of surface antigens (**B**). The data are representative of three separate experiments performed in duplicate.

of tumoral myeloid precursors along the granulocytic lineage. To this end, both NB4 and NB4-R cells were transiently transfected with a plasmid containing the full-length cDNA of human PLC-β2 connected with the cDNA for the EGFP (Fig. 7A), and cultured in control conditions or induced to differentiate in the presence of ATRA and As_2O_3, alone or in combination, at doses inducing low levels of differentiation. As shown in Figure 7B, the selective cytofluorimetric analysis of the EGFP-labeled green cells demonstrated that the sole overexpression of PLC-β2 did not induce modifications of CD11c expression in both control and agonist-treated cells.

DISCUSSION

A long tradition of arsenic compounds in Chinese medicine provided the basis for the formal introduction of As_2O_3 in the treatment of patients with APL. Now, this chemical can be used as an alternative or in concomitance with ATRA, particularly in patients with relapsed and refractory APL [Miller and Waxman, 2002; Miller et al., 2002; Slack et al., 2002; Lazo et al., 2003; Evens et al., 2004]. The mechanisms of As_2O_3 action are not fully understood, although it has been reported that As_2O_3 affects APL cells inducing both apoptosis and partial differentiation [Chen et al., 1997; Shao et al., 1998; Miller et al., 2002]. Studies on both patients and APL-derived cell lines indicate that low concentrations of As_2O_3 induce morphological changes of the leukemic cell population [Chen et al., 1997; Evens et al., 2004] and modulate the expression of cell surface differentiation antigens [Soignet et al., 1998]. On the other hand, high concentrations of As_2O_3 inhibit growth and reduce the viability of APL blasts and NB4 cells, mainly through apoptosis [Chen et al., 1997].

In our experiments, focused on the differentiating role of As_2O_3, we have demonstrated that the administration of low doses of this compound induced, in NB4 cells as well as in the NB4-R subclone, only partially responsive to ATRA, a minimal neutrophil-like differentiation, more evident in the retinoid-resistant cells. These data are in agreement with the in vivo results indicating that As_2O_3 exerts the best effects in treatment of patients refractory to ATRA-based therapies [Lazo et al., 2003; Tallman, 2004], and with in vitro experiments on APL-derived NB4 cells, sensitive, or resistant to ATRA [Jing et al., 2001].

Our previous work indicates that PLC-β2, highly expressed in neutrophils from peripheral blood, is nearly absent in promyelocytes from patients with APL. We have found that treatment with ATRA restores the amount of this PLC isozyme and that PLC-β2 represents a sensitive marker to monitor ATRA-induced granulocytic maturation of APL-derived malignant myeloid precursors [Bertagnolo et al., 2002]. In this work, we evaluated the expression of PLC-β2 when APL-derived cells were treated with As_2O_3 at differentiating concentrations, and we have found a low but significant dose-dependent increase of this protein, particularly evident in the NB4-R clone. Interestingly, at all the explored differentiating concentrations, the amount of PLC-β2 paralleled the differentiation levels reached by both ATRA-sensitive or partially resistant cell lines.

Studies performed on patients with newly diagnosed APL demonstrate that ATRA/arsenic combinations for remission and maintenance phases bring much better results than either of the two drugs used alone, in terms of quality of complete remission and status of the disease-free survival [Shen et al., 2004; Wang et al., 2004]. Accordingly, in a transgenic mouse model of APL, the combined administration of retinoic acid and arsenic trioxide enhances the regression of established leukemia [Lallemand-Breitenbach et al., 1999]. A synergism between ATRA and As_2O_3 in inducing granulocytic differentiation was also demonstrated in subclones of NB4 that are As_2O_3- or ATRA-resistant [Gianni et al., 1998; Jing et al., 2001].

Here we have demonstrated that differentiating doses of the two agonists cooperate not only to induce differentiation, in both ATRA sensitive and partially resistant cells, but also to induce PLC-β2 expression, that, particularly in ATRA-resistant cells, paralleled the differentiation levels at all the explored differentiating concentrations. These data indicate that the increase of PLC-β2 expression is not exclusive of the ATRA-activated maturation machinery, but is an event induced by different molecules, able to induce the overcoming of the differentiation block.

The existence of a synergism between ATRA and As_2O_3 in inducing the maturation of both ATRA-responsive and -resistant cell lines suggests that it may be possible to obtain elevated

differentiation levels also in the presence of reduced concentrations of the agonists, according with the most recent clinical trials that employ combinations of low doses of the two agonists to reduce the secondary effects of the drugs [Wang et al., 2004]. We found that in ATRA-responsive NB4 cells, the concentration of ATRA may be reduced 10-fold in the presence of low doses of As_2O_3, to obtain elevated differentiation levels, while the maturation achieved by the ATRA-resistant clone depends on the amount of the single drugs in the ATRA/As_2O_3 combination. At all the explored differentiating conditions, the amount of PLC-β2 increases in parallel with the maturation level, indicating that this molecule constitutes a sensitive marker for monitoring the release of the maturation block.

It has been reported that PML-RARα-bound co-repressors are released from DNA upon both ATRA and As_2O_3-treatment of APL cells leading to the activation of genes repressed by fusion protein [Denis et al., 2005]. This suggests that the reduced expression of PLC-β2, whose gene is located on chromosome 15, involved in the (15;17) translocation, may be related to the presence of the fusion protein. This hypothesis is substantiated by the fact that HL-60, an APL-derived cell line that does not possess the (15;17) translocation but differentiates after treatment with micromolar concentrations of ATRA, shows high PLC-β2 levels [Bertagnolo et al., 1997]. The increased expression of PLC-β2, induced by both ATRA and As_2O_3, may be related indeed to the removal of the fusion protein, that seems to constitute a common step of the differentiation pathways activated by the two agonists [Melnick and Licht, 1999; Zhu et al., 2001; Miller et al., 2002].

Since it has been reported that genes induced in common by ATRA and As_2O_3 are likely to be involved in myeloid differentiation [Zhu et al., 2001], and we have found that both ATRA and As_2O_3, alone or in combination, induce the expression of PLC-β2, the possible role for this protein in modulating maturation of APL-derived cells was investigated. Downmodulation of PLC-β2 during the differentiation process induced by the two agonists, alone or in combination, did not have significant effects on the differentiation levels reached by both NB4 and NB4-R cells.

We then tried to assess whether a forced expression of PLC-β2 is capable to promote differentiation or to improve the agonist-induced maturation of APL-derived cells, observing that an amplified expression of PLC-β2 did not induce a significant modifications of the reached maturation levels, under all the explored conditions. These data further suggest that the low amount of PLC-β2 in APL-derived cells is related to the presence of the transcriptional block due to the chromosomal translocation typical of this subtype of myeloid leukemia, rather than to the low differentiation level of tumoral promyelocytes. Our previous findings [Bertagnolo et al., 1997], demonstrating that elevated amounts of PLC-β2 are present in HL-60 cells, promyelocytes that are blocked to a poorly differentiated state, support this hypothesis. Accordingly, the total amount of PLC-β2 in HL-60 cells is not modified during differentiating treatment with ATRA [Bertagnolo et al., 1997].

This group of data indicates that PLC-β2, even though its expression closely correlates with the differentiation levels reached by APL-derived cells induced to complete their differentiation plan, is not able to activate a maturation programme and is not directly involved in the agonist-induced machinery ended to drive the precursors to the neutrophil-like stage.

Remarkably, our data demonstrate that an increased expression of PLC-β2 is not peculiar of the differentiative response of tumoral myeloid precursors to a single agonist, but it represents a specific step in the overcoming of the maturation block of APL-derived promyelocytes. This protein closely correlates with the differentiation level reached by APL-derived cells cultured in the presence of the drugs currently employed in treatment of APL patients. For this reason, it may constitute a marker to evaluate the patient responsiveness to As_2O_3 and As_2O_3/ATRA-based therapies, similarly to what we have already described for ATRA alone. Finally, PLC-β2 is a candidate molecule potentially useful to determine the efficacy of new drugs in releasing the block of gene transcription that characterizes acute promyelocytic leukemia.

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