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ORIGINAL ARTICLE

Production of functional platelet-like particles by the megakaryoblastic DAMI cell line provides a model for platelet biogenesis

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Abstract

The aim of this study was to evaluate cell maturation and the platelet production capacity of the megakaryoblastic DAMI cell line, to characterize platelet-like particles produced and to investigate the mechanisms involved in their production. DAMI cell maturation was induced by phorbol myristate acetate (PMA) and thrombopoietin (TPO). Expression levels of GATA-1, Fli-1 and NF-E2 were evaluated using real-time PCR and western blot. Platelet-like particles were characterized by the presence of GPIb and GPIIb by flow cytometry, while the soluble fragment of GPIb, glycocalicin, was detected by enzyme immunoassay. Dense and alpha granules were evaluated by mepacrine staining and thrombospondin-1 detection, respectively, and by electron microscopy. Functional capacity of platelet-like particles was studied by measuring P-selectin membrane after thrombin stimulation by flow cytometry and actin polymerization using phalloidin-FITC by immunofluorescence. We found that stimulation of DAMI cells with high concentration of PMA and TPO induced the expression of transcription factors GATA-1 and Fli-1 followed by an increase in the isoform a of NF-E2. Mature DAMI cells give rise to extensions resembling proplatelets and later, produce platelet-like particles expressing GPIIb and GPIb on their surface and containing dense and alpha granules, which were confirmed by electron microscopy. Platelet functionality was demonstrated by the increase in P-selectin membrane expression after thrombin stimulation and by their ability to spread on fibrinogen matrices. DAMI cell line induced to differentiate into mature megakaryocytes is able to produce functional platelets providing a suitable model to study the mechanisms involved in platelet generation.

Keywords: *Cell line, DAMI, NF-E2, platelet, thrombopoiesis*

Introduction

Megakaryopoiesis is the process that leads to platelet production. This process involves commitment of multipotent hematopoietic stem cells towards megakaryocyte progenitors, their proliferation, differentiation and polyploidization [1]. Cytokines and specific transcription factors are the main players in these events. Among the latter, GATA-1 and Fli-1 are involved in megakaryocyte differentiation and endomitosis, as revealed by murine models [2, 3]. Once megakaryocytes become mature, they produce platelets through a mechanism called proplatelet formation. This final step of platelet assembly and release represents a unique cellular transformation event that is orchestrated by transcription factors, signalling molecules and cytoeskeletal proteins. NF-E2 is the main transcription factor involved in this process [4, 5]. Cytoskeleton reorganization and active granule transport represent key events during proplatelet formation [6–8]. However, deep knowledge of this mechanism, including specific participating signaling molecules is still lacking. In vitro culture systems of megakaryocytic cell lines are a useful tool for these studies. Among them, Meg-01 has been used to assess platelet production and detailed characterization of the particles generated by this cell line has been performed [9]. However, in some others, such as DAMI cells, these particles have not been either isolated or characterized.

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The megakaryoblastic cell line DAMI is derived from the peripheral blood of a patient with megakaryoblastic leukaemia [10]. These cells have characteristics of megakaryoblasts or immature megakaryocytes, displaying many of the morphologic and biochemical features of the megakaryocytic lineage. DNA content is predominantly 2N and 4N while a small proportion of cells are 8N or greater. Cells have high nuclear-cytoplasm ratio and display glycoprotein (GP) Ib and GPIIb/IIIa membrane expression [10]. These cells can be induced to differentiate further along the megakaryocytic lineage by the tumor-promoting phorbol ester, phorbol myristate acetate (PMA) [10]. PMA-stimulated DAMI cells increase their ploidy and the expression of GP IIb/IIIa and Ib, both characteristic markers of the megakaryocytic membrane [10]. Briquet-Laugier et al. [11] have shown that stimulation with a high concentration of PMA together with thrombopoietin (TPO) were necessary to induce the generation of alpha granules in the cytoplasm of DAMI cells. However, the platelet production capacity of this cell line has not been investigated.

In the present study, we used a similar approach to induce cell maturation and to evaluate platelet production by DAMI cells. In addition, we characterized the structural and functional properties of the platelet-like particles produced and investigated the mechanisms involved in their generation.

Results show that stimulation of DAMI cells with high concentration of PMA and TPO induces the production of functional platelets, providing a suitable model to study the mechanisms involved in platelet generation.

Materials and methods

Reagents and antibodies

Phorbol myristate acetate (PMA), indomethacin, phalloidin-FITC, fibrinogen, prostaglandin E2 (PGE), DNAse, mepacrine, propidium iodide, Hoechst, anti β-actin and anti-thrombospondin-1 antibodies were from Sigma-Aldrich (St Louis, MO, USA). Recombinant human thrombopoietin (TPO) and thrombospondin-1 enzyme-linked immunosorbent assay were from R&D Systems (Minneapolis, MN, USA). Glycocalicin enzyme immunoassay was from TAKARA (Takara Bio Inc., Shiga, Japan). Mouse anti-human CD61-FITC (anti-GPIIIa), CD41a-PE (anti-GPIIb), CD42b-PE (anti-GPIb), CD62P-PE (anti-P-selectin), goat anti mouse IgG-FITC conjugated and their respective isotype controls were obtained from Becton Dickinson Biosciences (San Jose, CA, USA). Thrombin was from Diagnostica Stago (Asnières sur Seine, France). Iscove's modified Dulbecco's medium (IMDM), Trizol reagent and Super Script^{TM} preamplification system were obtained from Gibco-BRL (Grand Island, NY, USA). SYBR Green Supermix was from Bio-Rad (Hercules, CA, USA). TNFα protease inhibitor (TAPI) was obtained from Peptides International Inc (Kentucky, LO, USA). RNAase was from Roche Diagnostics Gmbh (Mannheim, Germany). Polyclonal antibodies against human GATA-1, Fli-1 and NF-E2 were from Santa Cruz Biotechnologies (CA, USA).

Cell culture

DAMI cells were a generous gift from Dr. M.C. Martyré, INSERM U602, Hopital Paul Brousse, Villejuif, France. The properties of the human megakaryoblastic cell line DAMI have been previously described [10]. DAMI cells were cultured in IMDM supplemented with 10% horse serum (Gibco) and penicillin-streptomycin (50 units/mL and 50 mg/mL, respectively, Gibco) at 37°C in a humidified atmosphere with 5% CO_2. DAMI cell differentiation was induced with 1 μM PMA and 10 ng/mL TPO during 7 days and cultured for another 3 days without stimulation to evaluate the characteristics of the particles produced. For some experiments, PMA concentrations ranging from 5 nM to 50 nM at a single dose were used. Cells were seeded in plastic tissue culture flasks or wells, at a concentration of 2.5×10^5/mL. Assays were made in triplicate.

Characterization of DAMI cells

Evaluation of membrane antigens. The expression of surface antigens was analysed using monoclonal antibodies recognizing glycoproteins that identify the megakaryocytic lineage. Stimulated DAMI cells were collected in IMDM medium and adjusted to a concentration of 1×10^5/mL. Cells were incubated with anti GPIIb (CD41a-PE), GPIb (CD42b-PE) and P-selectin (CD62P-PE). Non specific mouse IgGs were used as isotype controls. Cells were fixed with 1% paraformaldehyde and samples were acquired in a FACSCalibur flow cytometer (Becton Dickinson Biosciences). Results were expressed as percentage of positive cells (%) and relative fluorescence intensity (RFI) (specific binding/isotype control).

Ploidy analysis. Stimulated cells were collected, washed in PBS supplemented with 5 mM EDTA and 1% BSA. Propidium iodide (1 μg/mL), RNAase (100 μg/mL) and Tween X-100 (0.1%) were added and further incubated during 30 minutes. The reaction was stopped with PBS and ploidy was evaluated by flow cytometry within 30 minutes.

Immunofluorescence staining. Stimulated DAMI cells were transferred onto sterile glass coverslips coated with fibrinogen (200 μg/mL) and incubated at 37°C in a 5% CO_2 humidified atmosphere for 20 h. Cells were fixed with 3% paraformaldehyde, washed twice and permeabilized with 0.2% triton X-100. Between each of the following steps, cells were washed three times at room temperature. After a blocking step with 10% fetal calf serum and 1% BSA for 1 hour, cells were incubated either with phalloidin-FITC (10 μg/mL) or anti-GPIIIa (CD61) followed by a FITC-conjugated secondary antibody. After a washing step nuclei were stained with Hoechst (400 ng/mL). Coverslips were mounted with Prolong-Gold (Invitrogen) into microscope slides for fluorescence analysis using a fluorescence microscope (Carl Zeiss Microimaging Inc., NY, USA).

Expression of GATA-1, Fli-1 and NF-E2. Relative expression of these transcription factors was determined by real-time RT-PCR. Stimulated DAMI cells were harvested every 24 h during 10 days and unstimulated cells were used to determine basal expression levels. Total RNA was isolated using Trizol reagent from 1×10^6 cells according to Chomczynski and Sacchi [12]. DNA was digested with DNAse during 15 min at room temperature. cDNA was synthesized from 1 μg RNA using the Super Script™ preamplification system according to the manufacturer's instructions. Real-time PCR was performed using IQ™ SYBR Green Supermix in a iCycler thermocycler (Bio-Rad, CA, USA). Annealing temperatures were set at 64°C. Samples were run in triplicate and a negative control was included in each assay. Relative expression of target gene mRNA was normalized to the amount of a housekeeping gene (GAPDH mRNA). A fluorescence threshold cycle value (ct) was calculated for each sample. Levels of mRNA were expressed as the ratio between relative amounts of GAPDH and the target gene. Melting curve analysis and 2% agarose gel electrophoresis followed by ethidium bromide staining were performed to confirm the identity of PCR products.

Forward (f) and reverse (r) primer sequences for GATA-1 were: 5′-CTGGGAT CACACTGAGCTTGC-3', 5′-GATTAACCT GGGCTGGTGGTT-3'; F and R for Fli-1 were: 5′-CACCACCCTCTACAACACGGA-3', 5′-TTG GTCGGTGTGGGAGGTT-3'; F and R for aNFE2 were: 5′-CCTGCTGTGACTCCACC ACA-3', 5′-GCCAGAGTCTGGTCCAGGTTC-3' F and R for fNFE2 were: 5′TGACTCTGCCTTT AGCCAGGA3', 5′-CCAGATGGCTCTAGAAA CCTT-3', F and R for GAPDH were: 5′-ATCTTCCAGGAGCGAGATCC-3', 5′-CTGC AAATGAGCCCCAGCCT-3'.

Protein levels of GATA-1, FLi-1 and NF-E2. Dami cells were harvested and lysed in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) then supplemented with 2 μg/mL aprotinin, 2 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 2 μg/mL leupeptin and 0.1% SDS. Samples were centrifuged at 10,000 rpm for 30 minutes and proteins were separated by SDS/PAGE (12% gel) and blotted into nitrocellulose membrane. Polyclonal anti-human GATA-1, FLi-1, NF-E2 rabbit IgG and mouse β-actin were used. After washing, membranes were incubated with horseradish-peroxidase (HRP)-linked secondary antibodies anti-rabbit IgG and anti-mouse IgG, and detection was performed by enhanced chemiluminescence (Cell Signaling, MA, USA). Protein expression were normalized to β-actin using a densitometer (Bio-Rad).

Characterization of platelet-like particles production by DAMI cells

Quantification of platelet-like particles. Platelet-like particles were quantified in the supernatant of cultured cells by flow cytometry. Supernatants were collected, incubated with mouse anti-human CD41a-PE or CD61-FITC during 30 minutes and fixed in 1% paraformaldehyde. Platelet-like particles were identified by their size, granulation and their capacity to bind anti-CD41a or anti-CD61, using normal human platelets from peripheral blood as control. Particles were counted during 30 seconds in a continuous flow (100 μL/minute).

Expression of membrane antigens on platelet-like particles. Platelet-like particles were selected by their similarity to peripheral blood platelets considering size (FSC) and complexity (SSC). CD41a-PE and CD42b-PE were evaluated as described above for DAMI cells.

Glycocalicin measurement. Glycocalicin was quantified on culture supernatant of DAMI cell, collected on days 3, 5, 7 and 10. Samples were centrifuged at 10,000 rpm for 30 minutes. Glycocalicin was measured by enzyme immunoassay as recommended by the manufacturers; the lower detection limit is 10 ng/ml. All samples were assessed in duplicate.

Evaluation of dense granules content. Supernatant of DAMI cell culture was incubated with anti-CD41a-PE and 40 μM mepacrine for 30 minutes. Mepacrine staining was evaluated in CD41a-positive platelet-like particles by flow cytometry.

Table I. Glycoprotein expression in DAMI cells membrane.

Glycoprotein expression	Unstimulated DAMI cells		DAMI cells + PMA and TPO	
	%	RFI	%	RFI
GP IIb	47 (35–52)	2.62 (2.1–3.01)	57 (41–66)	5.81 (2.3–6.75)
GP Ib	47 (39–48)	2.39 (2.16–3.2)	21 (8.5–33)*	1.75 (1.3–3.9)
P-selectin	6.85 (2.8–10.5)	1.45 (1.15–1.9)	11.7 (8.2–13.7)*	1.67 (1.2–1.8)

*$p = 0.035$, unstimulated vs. stimulated DAMI cells. Percentage of positive cells and relative fluorescence intensity in stimulated (PMA plus TPO) and unstimulated cells are shown. Results are expressed as median and range of three independent experiments.

Measurement of thrombospondin-1. Thrombospondin-1 was evaluated as a marker of alpha granules. Supernatant of DAMI cell culture, collected every two days during 10 days, was centrifuged at 10,000 rpm for 30 minutes and stored at −70°C until use. Thrombospondin-1 release was measured in the culture supernatant by enzyme-linked immunosorbent assay, as recommended by the manufacturers; the lower detection limit is 0.355 ng/mL. All samples were assessed in duplicate. Thrombospondin-1 content in platelet-like particles was also detected by immunofluorescence as described above for phalloidin-FITC staining with a mouse anti-Thrombospondin-1 antibody (10 μg/mL) and a secondary goat anti-mouse IgG FITC-conjugated antibody (16 μg/mL).

Electron microscopy. PMA-stimulated samples containing platelet-like particles were collected and fixed in 0.1 M phosphate buffer, 5% Glutaraldehyde (Sigma Chemical Co, St Louis, USA), pH 7.2, rinsed in the same buffer, postfixed in Osmium tetroxide (Sigma Chemical Co) and embedded in Araldite (Sigma Chemical Co). Thin sections were stained with Uranil acetate (Riedel-d Haen, AG, Alemania) and lead Citrate (Sigma Chemical Co), and examined in a transmission electron microscope (Zeiss, EM 900, Germany).

Functional studies of platelet-like particles

P-selectin expression. Platelet-like particles were incubated with or without 2 U/mL thrombin and labeled with CD61-FITC and CD62P-PE antibodies. After fixation with 1% paraformaldehyde, the expression of CD62P was analysed in the CD61-positive population by flow cytometry.

Actin polymerization. Actin polymerization was analysed after platelet spreading on immobilized fibrinogen. Platelets were incubated for 20 minutes on slides previously coated with 200 μg/mL fibrinogen. After fixation with 1% paraformaldehyde, samples were treated with acetone and the actin filaments were stained with 10 μg/mL phalloidin-FITC. Platelet spreading was observed by fluorescence microscopy.

Statistical analysis

Results were expressed as median and range. Student *t* test was used to compare glycoprotein expression between unstimulated and stimulated DAMI cells. Wilcoxon test was used to compare P-selectin expression in platelet-like particles with or without thrombin stimulation.

Results

DAMI cells characterization

The megakaryoblastic characteristics of this cell line have been widely described [10]. Membrane glycoprotein IIbIIIa and Ib expression in unstimulated cells is shown in Table I. Expression of P-selectin, an alpha-granule membrane protein, was observed on the membrane in a small percentage of unstimulated DAMI cells (Table I). Ploidy values in unstimulated cells were predominantly 2N and 4N, with a small proportion of 8N or higher (2N 66%; 4N 33%; 8N 1%; median of three independent experiments), as described previously [10]. In accordance with these results, scarce nuclei lobulation was observed by immunofluorescence microscopy. These cells spontaneously released a small number of platelet-sized particles into the culture medium, 3670 (1000–6000) particles/mL when grown at 300.000 cells/mL.

In order to induce megakaryocytic differentiation, a scheme of daily stimulation with 1 μM PMA and 10 ng/mL TPO during 7 days, followed by 3 days without the addition of any drug was used. Subsequent experiments were performed at day 10 of culture. Stimulated DAMI cells displayed a significant decrease in their proliferation rate, and an increased adherence to the plastic culture plates and cell spreading, as previously described [10]. A non-significant increase in GPIIb expression ($p = 0.28$), as well as a significant reduction in GPIb expression ($p = 0.035$), were observed on day 10 of culture. Moreover, when tested along the culture period, GPIb expression progressively decreased on cell membrane (data not shown). Besides, P-selectin increased significantly compared

to unstimulated DAMI cells ($p = 0.035$) (Table I). Polyploidization was also increased in response to PMA plus TPO, reaching 8N or higher in 17% of the cell population (2N 50%; 4N 33%; 8N 7.4%; 16N 3.5%; 32N 2.8%; 64N 2%; 128N 1.3%; median of three independent experiments).

Expression levels of GATA-1, Fli-1 and aNF-E2 during DAMI cell maturation

We evaluated the expression of transcription factors involved in MK maturation and platelet formation in DAMI cells. Relative expression of specific mRNAs was determined by real-time PCR. The kinetics of GATA-1 and Fli-1, the main transcription factors involved in differentiation and endomitosis of megakaryocytic progenitors, was studied. Both GATA-1 and Fli-1 mRNA levels increased after PMA-TPO stimulation on days 2 and 1, respectively (Figure 1A, B) and a second peak was evident between days 5 and 7, decreasing to basal levels at day 10 for GATA-1, but remaining moderately elevated for Fli-1. At the protein level, GATA-1 showed an increase on day 2 in agreement with mRNA levels, and remained high until the end of cell maturation (Figure 1C). Fli-1 protein presented two waves of increase in accordance with mRNA fluctuations (Figure 1D). Expression of NF-E2, the main transcription factor related to platelet formation, was also evaluated. NF-E2 has two mRNA isoforms, aNF-E2 and fNF-E2, although the relative contribution of both isoforms to thrombopoiesis has not yet been established. We found a progressive increase in the expression of aNF-E2 isoform until day 6, followed by a second peak on day 9, while fNF-E2 isoform decreased over time in stimulated DAMI cells, with minor fluctuations in its expression levels along the culture period (Figure 1E, F), suggesting that aNF-E2 is the main isoform related to thrombopoiesis. At the protein level, an increase in NF-E2 was observed from day 5 to day 10 in agreement with a rise in platelet-like production (Figure 1G). Western blots for transcription factors and β-actin are shown in Figure 1H.

Stimulated DAMI cells produce platelet-like particles

In order to evaluate the platelet-sized particles produced by this cell line, CD61-positive events with similar size and complexity (forward and side scatter) to peripheral blood platelets were analysed (Figure 2A, B). A dose and time-dependent increase in platelet-like particles production was observed when cells were stimulated with PMA. Moreover, addition of TPO rendered a higher number of platelet-like particles (Figure 2C).

Characterization of platelet-like particles

We evaluated the presence of lineage-specific surface glycoproteins on platelet-like particles produced by PMA and TPO-stimulated DAMI cells by flow cytometry. GPIIb expression was observed in 29% (17–36) of the platelet-like particles derived from DAMI cells, RFI: 2 (1.4–2.55) (Figure 2D). GPIb was expressed in 12.8% (2–19.6) of GPIIb-positive particles, RFI: 1.32 (1–2.2).

In order to evaluate whether the low expression of GPIb was inherent to platelet-like particles or due to the loss of this membrane glycoprotein during the culture period, time-course GPIb expression on particle membrane was evaluated. GPIb expression decreased from day 5 to day 10 as shown in Figure 2E. We considered in vitro shedding as a possible cause of GPIb decrease. In order to test this hypothesis, the soluble fragment of GPIb, glycocalicin, was measured over time in stimulated DAMI cell supernatant. In addition, GPIb expression was evaluated 24 h after the addition of TAPI, a TNFα metalloprotease inhibitor (200 μmol/L), at day 10 of culture [13]. Glycocalicin concentration increased along the culture period, as shown in Figure 2F. Besides, in the presence of TAPI an increase from 19.6% to 27.7% in GPIb expression was observed. Altogether, these results suggest GPIb proteolytic cleavage during culture.

Platelet-like particles contain dense and alpha granules

Evaluation of dense granules. Platelet-like particles were incubated with mepacrine to evaluate their dense granule content. DAMI cells stimulated with PMA alone produced particles that were not stained by mepacrine (RFI: 1.13), indicating lack of dense granules. However, when stimulated with 1 μM PMA plus TPO, DAMI cells produced particles able to be stained by mepacrine, although with lower fluorescence intensity than peripheral blood platelets, RFI: 2.4 (1.8–3.15) and RFI 4.54 (3.25–6.33), respectively (Figure 3A).

Evaluation of alpha-granules. The presence of alpha-granules in DAMI cells and platelet-like particles was evaluated by measuring thrombospondin-1 release into the cell culture supernatant and by immunofluorescence in platelet-like particles. Soluble thrombospondin-1 was not detected in conditioned media from unstimulated DAMI cells. However, its levels increased progressively until day 10 of culture during PMA-TPO stimulation (Figure 3B). Immunofluorescent detection of thrombospondin-1 showed a granular pattern in platelet-like particles, similar to that found in peripheral blood platelets (Figure 3C, D).

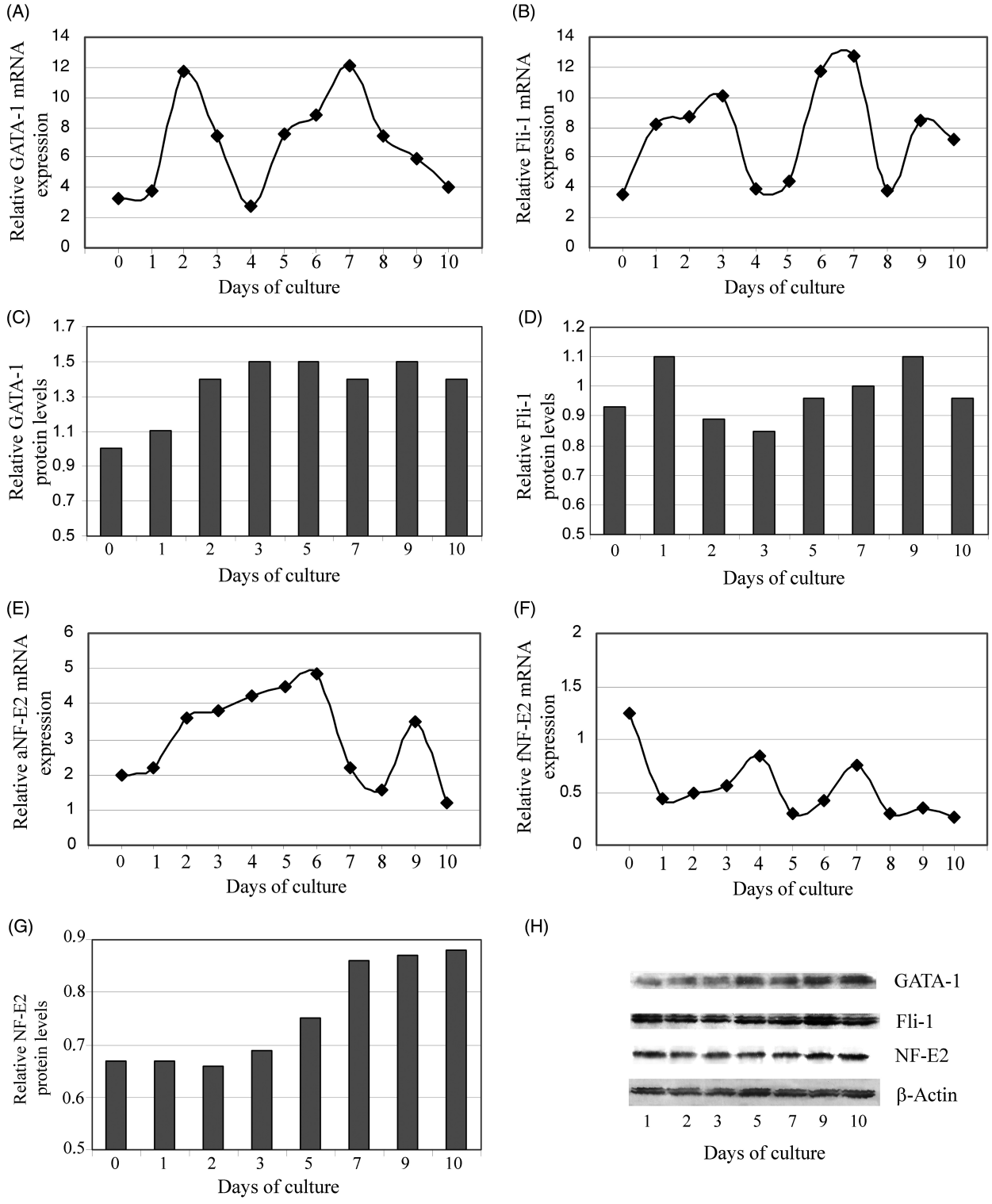

Figure 1. Expression levels of GATA-1, Fli-1, aNF-E2 and fNF-E2 transcription factors. RNA from PMA and TPO-stimulated and unstimulated DAMI cells was evaluated by real-time PCR along the culture period (A, B, E, F). Specific mRNA levels were expressed as the ratio of GAPDH mRNA and that of each target gene. Western blot analysis of protein transcription factors normalized to β-actin along the culture period (C, D, G). Representative example of western blots (H).

Electron microscopy. DAMI cells stimulated with PMA alone displayed immature cytoplasmatic organization and produced platelet-like particles lacking granules. However, when stimulated with 1 μM PMA and TPO, mature DAMI cells gave rise to platelet like-particles containing dense and alpha granules as shown by electron microscopy (Figure 4).

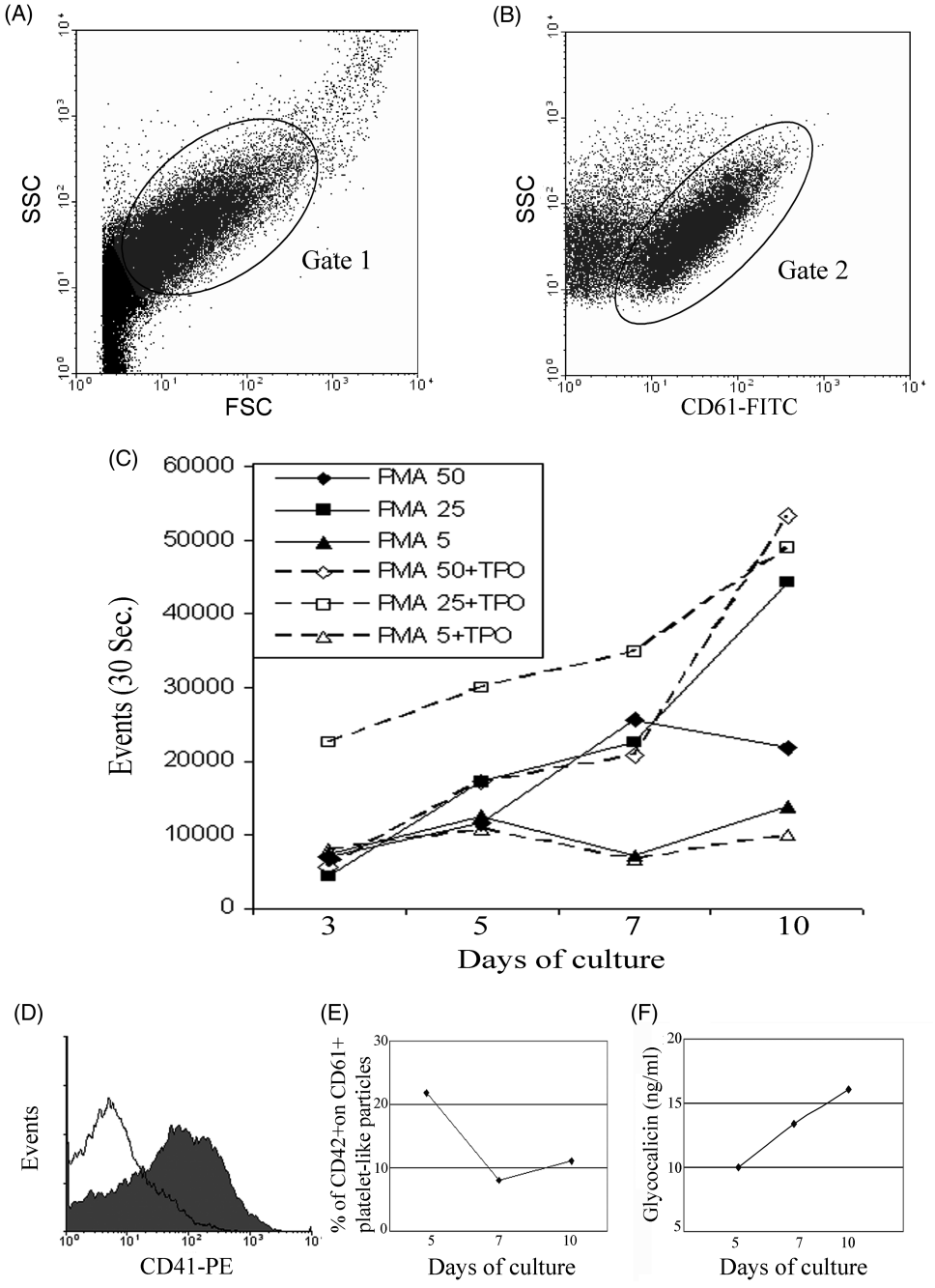

Figure 2. Platelet-like particles produced by stimulated DAMI cells. (A) Particles were collected from DAMI cell-culture supernatant, incubated with mouse anti CD61-FITC antibody and analysed by flow cytometry. Events included in Gate 1 were selected according to their similarity to peripheral blood platelets, considering complexity (forward scatter) and size (side scatter). (B) CD61-positive events (Gate 2) were selected from Gate 1 and enumerated. (C) Particles produced by DAMI cells stimulated with increasing PMA concentration (5–50 nM) with or without TPO, were collected along the culture period and incubated with CD61-FITC antibody. CD61-positive events were counted by flow cytometry, as described in Materials and methods. (D) Platelet-like particles obtained from DAMI cells stimulated with 1 μM PMA and TPO were collected on day 10 of culture, GPIIb expression was evaluated from Gate 1 using a specific antibody, CD41a-PE. Open histogram represent platelet-like particles labeled with the isotype control antibody and filled histogram, specific staining. A representative experiment is shown. (E) CD61 positive platelet-like particles were tested for GPIb at days 5, 7 and 10 of culture by labelling with CD42b-PE antibody. (F) Glycocalicin concentration was measured by enzyme immunoassay kit in the supernatant of stimulated DAMI cells.

Functional studies of platelet-like particles

Membrane P-selectin expression. Particles produced by PMA and TPO-stimulated DAMI cells were incubated in the presence or absence of thrombin to determinate their activation capacity by evaluating P-selectin membrane expression. P-selectin membrane expression was 16% (9.5–33), RFI: 1.65

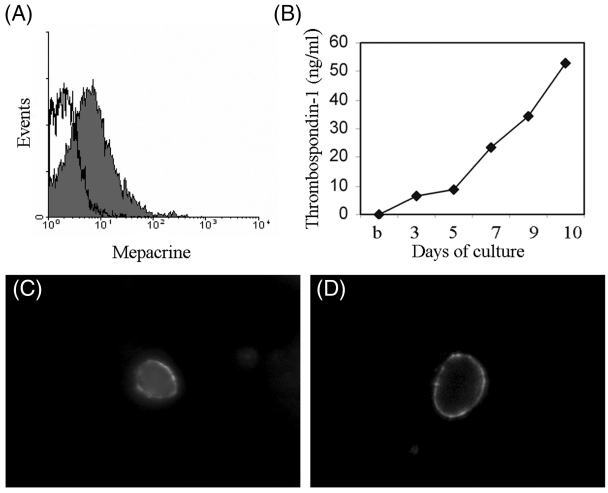

Figure 3. Evaluation of platelet-like particles granules. (A) CD41-PE positive particles were labeled with mepacrine to evaluate the presence of dense granules. Open histogram represent platelet-like particles incubated with the isotype control antibody and filled histogram, specific staining. (B) Thrombospondin-1 release was measured in the supernatant of DAMI cells along the culture period by ELISA. DAMI cells were seeded at 2.5×10^5/mL in IMDM medium. Values are expressed as ng/mL. b: represent conditioned media from unstimulated cells. (C, D) Thrombospondin-1 detection in platelet-like particles by immunofluorescence microscopy. Particles were stained with mouse anti-thrombospondin-1 IgG and visualized using FITC-conjugated anti-mouse antibody. (Original magnification × 1000).

(1.27–2.9) in resting conditions. After thrombin stimulation, a significant increase in P-selectin expression was observed, 22.45% (19.2–38.4) ($p = 0.0048$), RFI 2.36 (1.8–4).

In order to determine whether spontaneous particle activation occurred during the culture period, the same experiments were performed in the presence of platelet activation inhibitors (1 nM PGE, 15 ng/ml indomethacin) added 24 h before their collection. Under these conditions, P-selectin expression was 3% in resting conditions and 19% after thrombin activation. These data suggest there is spontaneous particle activation during culture that, when prevented by the addition of platelet activating inhibitors, unveils a greater functional capacity of these particles.

Actin polymerization in platelet-like particles and DAMI cells. In this study, the actin-binding toxin phalloidin-FITC was used to visualize actin polymerization in platelet-like particles derived from stimulated cells. Particles were allowed to spread on a fibrinogen-coated surface. Stimulated DAMI derived particles displayed lamellipodia and stress fibers, similar to peripheral blood platelets (Figure 5A). Interestingly, the proportion of platelets displaying morphological changes in relation to platelet activation was higher in the supernatant of DAMI cells than in peripheral blood, suggesting basal activation during culture.

Concerning the mechanism of platelet-like particles production, we were not able to observe typical proplatelet formation in stimulated DAMI cells either in suspension or after adhesion to fibrinogen matrices, as can be seen in megakaryocytes derived from human cord blood and peripheral blood CD34+ cells [14, 15] or murine megakaryocytes [5]. Instead, cytoplasmatic extensions with swellings resembling proplatelets were observed (Figure 5B). We were not able to see branching along the process or typical tips at the ends.

Discussion

In a number of studies the DAMI cell line has been used as a tool for the study of megakaryopoiesis and granule biogenesis [11, 16]. O'Brien et al. [17] have described the production of platelet-like particles by DAMI cells after stimulation with 15-deoxy-$\Delta^{12,14}$-PGJ2, although characterization of produced particles was not performed. Here, we describe specific megakaryocytic transcription factors and membrane protein expression in stimulated DAMI cells, as well as the structure and functional properties of the platelet-like particles produced. The time course and expression pattern of transcription factors involved in different stages of megakaryocyte differentiation and platelet production were evaluated in DAMI cells induced to differentiate with PMA and TPO. Expression of GATA-1 and Fli-1, both transcription factors involved in megakaryocytic differentiation and endomitosis, increased during the first days of DAMI stimulation leading to the rise of their protein levels, and, in accordance to the morphological changes observed, including polyploidization and maturation. In addition, the kinetics of NF-E2, a platelet production-related transcription factor, was also studied. NF-E2 has two mRNA isoforms derived from alternative promoters, aNF-E2 and fNF-E2, both producing the same NF-E2 protein [18]. Although expression of both NF-E2 isoforms is induced during megakaryocyte differentiation of cord blood CD34+ cells [19], the relative contribution of both isoforms to thrombopoiesis has not yet been established. In this study, fNF-E2 decreased along the stimulation period. This observation is in line with data from lineage-specific expression studies, which reveal that fNF-E2 is down-regulated when bipotential erythroid-megakaryocyte cell lines are induced to differentiate along the megakaryocytic lineage after PMA stimulation, suggesting that the f isoform has erythroid-specificity [19]. Our results showed that, contrary to fNF-E2, aNF-E2 isoform increased during PMA stimulation,

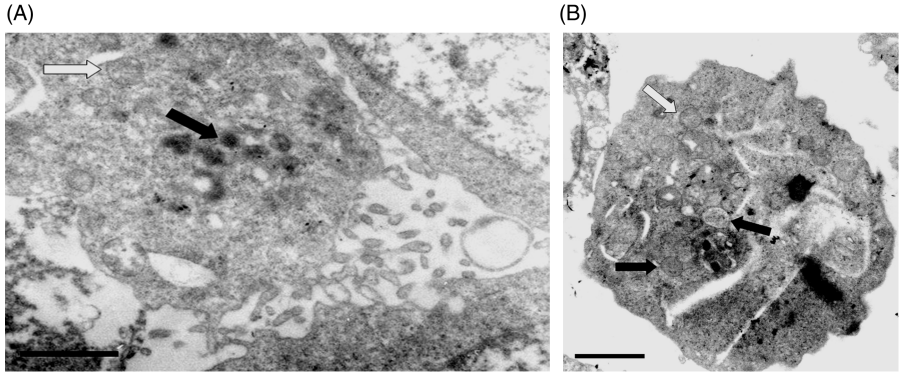

Figure 4. Electron microscopy of platelet-like particles. After 10 days of PMA and TPO stimulation particles produced by DAMI cells were prepared as described in Materials and methods, and photographed. Representative microphotographs of platelet-like particles are shown. Cytoplasmic organelles such as mitochondria (white arrows) are shown in (A) and (B). Dense granules (A) and alpha granules (B) can be observed (black arrows). Scale bars indicate 1.1 μm.

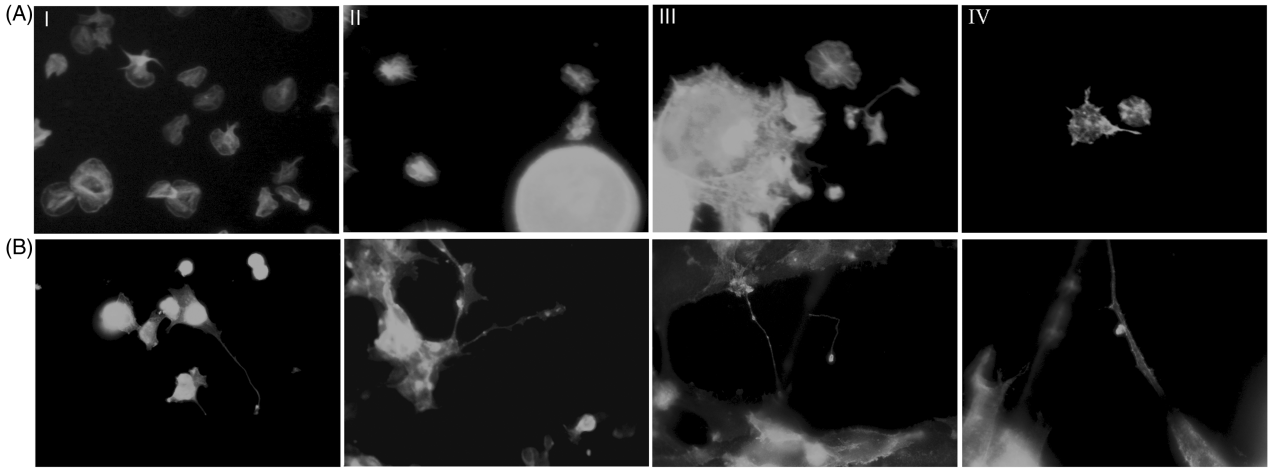

Figure 5. (A) Actin polymerization in platelet-like particles. Particles were allowed to spread on fibrinogen-coated coverslips, fixed with paraformaldehyde, permeabilized and stained with Phalloidin-FITC. (I), platelets obtained from peripheral blood. (II–IV), platelet like-particles obtained from stimulated DAMI cells after 10 days culture (Original magnification × 1000). (B) Stimulated DAMI cells were allowed to adhere on fibrinogen-coated coverslips, fixed, permeabilized and stained with anti-CD61 and a secondary conjugated-FITC antibody. Cytoplasmatic extensions resembling proplatelets are shown. Photographs are representative examples of three independent experiments. (Original magnification × 400).

reaching its highest expression levels later than those of GATA-1 and Fli-1, suggesting its role in thrombopoiesis in this cell line.

Preliminary experiments were carried out to evaluate the platelet production capacity of this cell line. In our experience, highest platelet particle production with lowest cell death was reached with 25 nM PMA and TPO. Particles produced under this condition were similar in size, morphology and membrane glycoprotein expression to peripheral blood platelets. However, neither cells nor platelet-like particles displayed megakaryocytic and platelet subcellular organization (i.e. mitochondria, alpha and dense granules), as observed by electron microscopy. Similarly, we could not detect dense granules in these platelet-like particles by mepacrine staining (data not shown). On the contrary, stimulation with 1 μM PMA plus 10 ng/mL TPO induced higher cytoplasmatic cell maturation. Indeed, the use of a tumor promoter such as PMA does not represent a physiologic stimulus but mimics those necessary to complete megakaryocyte maturation, which is an important feature in order to use this model to study the mechanisms of thrombopoiesis. Under these conditions, platelet-like particles contained alpha and dense granules, as demonstrated by thrombospondin-1 and mepacrine staining, respectively, which was confirmed by electron microscopy. The functional properties of particles produced in vitro were shown by P-selectin surface expression in response to thrombin. Interestingly, P-selectin surface expression was found, albeit at lower levels, in a small percentage of platelet-like particles in resting conditions. The expression of P-selectin on platelet-like particles in the

absence of platelet-activating agents observed in this study together with the spontaneous release of thrombospondin-1 to the culture medium and the actin polymerization pattern characteristic of platelet activation, suggests the presence of basal platelet-like particle activation. This basal stimulation could be due to the action of PMA, which activates PKC, a well-known mediator of platelet activation [20]. The fact that addition of platelet activation inhibitors such as PGE and indomethacin decreased P-selectin basal expression supports this hypothesis.

We found a progressively decrease in GPIb expression on stimulated DAMI cells membrane, as well as in platelet-like particles along the culture period. The concomitant increase in Glycocalicin levels in culture supernatant and the increase in membrane GPIb expression found in the presence of the metalloprotease inhibitor TAPI indicates that this decrease could be due to in vitro metalloprotease ADAM17 activity [21]. Interestingly, similar results were obtained by Nishikii et al. [22] in platelets generated from mouse embryonic stem cells-derived megakaryocytes, showing that addition of metalloprotease inhibitors to the culture system increased GPIb expression.

Actin filament organization in platelet-like particles allowed to adhere to fibrinogen-coated coverslips showed the same pattern as peripheral blood platelets, indicating that particles produced by this cell line are able to undergo cytoskeleton reorganization in response to extracellular stimuli that trigger outside-in signaling, thus providing further evidence for the functional capacity of these particles.

The mechanism by which DAMI cells produce platelets is not evident. Although cytoplamic extensions reminiscent of proplatelets were seen, these structures differed from typical proplatelets, as they showed no branching and lacked the characteristic tips at the ends. The adherent properties of stimulated DAMI cells could affect the structure of proplatelets in these cells. Alternatively, the mechanism of platelet shedding by DAMI cells could differ from that of human or mouse primary megakaryocytes. Other potential mechanisms of platelet release, such as cytoplasmatic budding or fragmentation could hypothetically take place in this case.

Since the discovery of thrombopoietin, several in vitro culture systems have been developed to study the mechanisms underlying megakaryopoiesis and proplatelet formation, including human primary megakaryocytes derived from CD34 progenitor cells, human megakaryocytic cell lines, murine fetal liver megakaryocytes and, more recently, murine embryonic stem cells induced to differentiate along the megakarocyitc lineage [1–5, 22, 23]. The combined use of these different experimental models may provide additional information that may contribute to current knowledge in megakaryocyte and platelet biology. Platelet generation by DAMI cells represents another tool to study certain aspects of the mechanisms underlying platelet production.

In summary, we show that DAMI cells stimulated with high PMA concentrations and TPO release functional platelet-like particles possibly under the control of NF-E2 isoform a. These particles show features resembling peripheral blood platelets, such as alpha and dense granule content, GPIIbIIIa and GPIb membrane expression. The functional capacity of these particles is revealed by their ability to be activated by thrombin and to adhere and spread on fibrinogen matrices. Considering that DAMI cells are relatively easy to handle and readily available, and are capable of yielding functional platelets, this study provides a simple alternative model to study platelet biogenesis and biology.

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