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Research Article

Characterization of Plasminogen Binding to NB4 Promyelocytic Cells Using Monoclonal Antibodies against Receptor-Induced Binding Sites in Cell-Bound Plasminogen

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The NB4 promyelocytic cell line exhibits many of the characteristics of acute promyelocytic leukemia blast cells, including the translocation (15 : 17) that fuses the PML gene on chromosome 15 to the RARα gene on chromosome 17. These cells have a very high fibrinolytic capacity. In addition to a high secretion of urokinase, NB4 cells exhibit a 10-fold higher plasminogen binding capacity compared with other leukemic cell lines. When tissue-type plasminogen activator was added to acid-treated cells, plasmin generation was 20–26-fold higher than that generated by U937 cells or peripheral blood neutrophils, respectively. We found that plasminogen bound to these cells can be detected by fluorescence-activated cell sorting using an antiplasminogen monoclonal antibody that specifically reacts with this antigen when it is bound to cell surfaces. All-*trans* retinoid acid treatment of NB4 cells markedly decreased the binding of this monoclonal antibody. This cell line constitutes a unique model to explore plasminogen binding and activation on cell surfaces that can be modulated by all-*trans* retinoid acid treatment.

1. Introduction

Interaction of components of the plasminogen system with fibrin or extracellular matrix promotes plasminogen activation [1]. In a similar way, when components of the plasminogen system are bound to cell surfaces, plasmin generation is increased [2–4]. Binding of plasminogen to cell surfaces is the most important event in the enhancement of plasmin-mediated pericellular proteolysis. Plasminogen binding has two major consequences: (1) plasminogen activation by either tissue-type plasminogen activator (tPA) or urokinase (uPA) is enhanced when plasminogen is bound to cells [5, 6] and (2) plasmin generated on the cell surface is protected from α_2-antiplasmin [4, 7], arming the cell with a more efficient proteolytic activity [5]. The promotion of plasminogen activation by cells can be explained by kinetic interactions with and on the cell surface, conformational effects, and/or receptor occupancy [5].

The amount of plasminogen bound to cell surfaces can be quantified. By using radiolabeled plasminogen, it is possible to detect specific binding of plasminogen to cells and to calculate binding affinities and the number of plasminogen binding sites. With this approach, a wide number of cell types have been analyzed (reviewed in [8]). In humans, platelets and all nucleated cells bind plasminogen with a Kd in the 0.3 to 2.8 μM range in agreement with the plasminogen concentration in plasma (1-2 μM). Cells exhibit a high capacity for plasminogen ranging from 10^4–10^5 binding sites for most nontransformed cells to 10^5–10^7 for malignant cells and human umbilical vein endothelial cells. In general, plasminogen binding capacity is paralleled by the capacity of the cells to promote plasminogen activation. For

example, in studies of plasminogen receptor modulation, it has been demonstrated that upmodulation of plasminogen binding sites, a process that can be induced by several effectors, induces an increase in the promotion of plasmin generation by cells [9]. Among malignant cells, the study of fibrinolysis on acute promyelocytic leukemia (APL) cells offered new insights in the understanding of this disease and its hemorrhagic complications.

APL is due to a clonal proliferation of promyelocytic blast cells carrying the $t(15:17)$ that fuses the PML gene on chromosome 15 to the RARα gene on chromosome 17 [10, 11]. In contrast to other leukemic processes, APL onset is frequently associated with life-threatening bleeding complications due to disseminated intravascular coagulation, abnormal fibrinolysis, or both [10–12]. Immature promyelocytes secrete high amounts of uPA [13, 14] that can promote plasmin formation *in vivo* and cause abnormal bleeding. In addition, a mechanism of promotion of plasminogen activation by cell surfaces has been explored on APL blast cells and on the promyelocytic cell line NB4. This promyelocytic cell line carries the typical translocation found in APL blast cells and has been used in a wide variety of biological studies on APL. The introduction of all-*trans* retinoid acid (ATRA) in the treatment of this disease in the nineties has dramatically changed the outcome of APL. In most APL patients, ATRA treatment induces differentiation of immature promyelocytic cells and corrects bleeding disorders. ATRA has several dramatic effects on the hemostatic system on both APL blast cells and on NB4 cells [15].

In this study, we sought to characterize plasminogen binding to NB4 cells using three different approaches. First, using radiolabeled plasminogen, we analyzed the plasminogen binding capacity of NB4 cells compared with other leukemic cells lines of different lineages. Second, we explored the functional consequences of plasminogen activation on NB4 cell surfaces analyzing plasmin generation by these cells. Finally, we measured plasminogen bound to this cell line by fluorescence-activated cell sorting analysis using an antiplasminogen monoclonal antibody that specifically interacts with plasminogen bound to cell surfaces [16] and explored the effect of ATRA treatment of NB4 cells on plasminogen binding.

2. Material and Methods

2.1. Proteins, Protein Iodination, and Antibodies. Glu-plasminogen was obtained from Chromogenix (Mölndal, Sweden). tPA (Actilyse) and high-molecular-weight uPA were obtained from Boehringer Ingelheim and Roger Laboratories (Molins de Rei-Barcelona, Spain), respectively. Glu-plasminogen was radiolabeled using a modified chloramine T method [17]. The labeled and unlabeled preparations of plasminogen used in this study had the characteristics of previously described preparations from our laboratory [17–20]. Antiplasminogen monoclonal antibody 49 (mAb49) was raised and characterized as previously described [16]. Fluorescein isothiocyanate (FITC) conjugated goat anti-mouse monoclonal antibodies were from Sera-Lab, Ltd.

2.2. Cells. Neutrophils, monocytes, and lymphocytes were isolated from blood collected into heparin (5 U/mL) as described [21]. NB4 cells were provided by Dr. M. Lanotte (Hôpital St. Louis, Paris, France). The human cell line, Nalm6, was provided by Dr. J. Inglés-Esteve (IDIBELL, Barcelona). Other cell lines were from the American Type Tissue Culture Collection (ATCC) and cultured in RPMI-1640 (Bio-Whitakker/MA Bioproducts) containing 1 mM Na pyruvate and 5–10% fetal bovine serum. Blast cells from peripheral blood were analyzed from a patient with acute nonlymphoblastic leukemia (ANLL), categorized according to the FAB classification [22].

2.3. Ligand Binding Analyses. Ligand binding analyses were performed as previously described by separating bound from free ligand by centrifugation over 20% sucrose [17–20]. Molecules of ligand bound per cell were calculated based on the specific activities of the radiolabeled ligands.

2.4. Cell-Dependent Promotion of Plasminogen Activation. Plasminogen activation studies were carried out in microtitre plates in reaction volumes of 100 μL as previously described [5, 6]. Briefly, 20 μL of plasminogen activators (tPA or uPA) (final concentration 70 and 37 pmol/L, resp.) were mixed with 40 μL of cells (final concentration 1.5×10^6 cells/mL) and 40 μL of substrate mix containing Glu-plasminogen (final concentration 100 nmol/L) and chromogenic substrate S-2251 (Val-Leu-Lys-p-nitroanilide; Chromogenix) (final concentration 0.15 mmol/L). Reactions were performed in assay buffer consisting of Tris-HCl, pH 7.4, at 37°C, and a final ionic strength of 0.12, containing 1 mg/mL human serum albumin. Absorbance was monitored at 405 nm, using a Thermomax thermostatted plate reader (Molecular Devices Corporation, Stanford, CA). Rates of plasmin generation were calculated as previously described [5, 6].

2.5. Fluorescence-Activated Cell Sorting (FACS) Analysis. Cells were washed with PBS containing 1% BSA and 0.1% sodium azide (PBA), incubated with PBA containing 10% heat-inactivated normal rabbit serum, washed again, and incubated with mAb49 (130 nM) or isotype control, washed, and then stained with FITC-goat anti-mouse IgG, which was detected in a flow cytometry analyzer (Coulter's EPICS XL-MCL). Plasminogen binding to cells in whole peripheral blood collected into EDTA was determined as above with the following exceptions. Cells were incubated in 10% heat-inactivated human AB serum in PBS, washed with PBA and incubated with anti-mouse IgG conjugated to PE, washed and incubated with FITC-conjugated antibodies to specific leukocyte antigens. Cells were incubated in Ortho-mune Lysing Reagent (Ortho Diagnostic Systems Inc.), centrifuged, and resuspended in PBA containing 7-aminoactynomycion D (Molecular Probes) at 1 mg/mL.

2.6. Reagents. Heparin, Tween 80, Tween 20, ε-ACA, and bovine serum albumin were from Sigma (St. Louis, MO). All-*trans* retinoic acid was from Hoffmanm-La Roche.

Table 1: Plasminogen binding to cell surfaces of several leukemic cell lines and normal peripheral blood cells.

Cell type	Cell lineage	Molecules of plasminogen bound/cell ($\times 10^6$)
NB4	Promyelocytic	38.0 ± 1.8
KG1a	Myeloblastoid	1.5 ± 0.2
K562	Erythromyeloid	3.1 ± 0.2
HL-60	Promyeloid	1.7 ± 0.3
U937	Monocytoid	2.0 ± 0.7
THP-1	Monocytoid	1.6 ± 0.2
Nalm6	Pre-B-Cell	1.2 ± 0.2
Molt4	Undifferentiated T cell	1.1 ± 0.4
	Neutrophils	0.35 ± 0.07
	Monocytes	0.58 ± 0.08
	Lymphocytes	0.59 ± 0.06

Binding analyses were carried out by incubation of washed cells (2–5 × 10^6/mL) with radiolabeled plasminogen (100 nM) in a total volume of 200 μL for 2 hours a 4°C. Cells were then separate from the whole reaction mixture by centrifugation of aliquots in 20% sucrose solution. The specific binding of radiolabeled plasminogen was determined by subtracting counts bound in the presence of 0.15 M ε-ACA. Results are the mean ± SD of 2–4 separate experiments.

Neutrophils, monocytes, lymphocytes, and RBC were isolated from blood collected into heparin (5 U/mL), theophylline (10 mM), and prostaglandin $E_{1\alpha}$ (10 U/mL) (Sigma) as described [6].

3. Results

3.1. Plasminogen Binding Capacity of NB4 Promyelocytic Cells. To explore the plasminogen binding capacity of this cell line with respect to other leukemic cells lines and peripheral blood cells, radiolabeled plasminogen (100 nmol/L) was added to washed cells (2–5 × 10^6/mL) and incubated for 1 hr at 37°C. After incubation, free ligand was separated by centrifugation on 20% sucrose. Specific binding was calculated by subtracting counts bound in the presence of 0.15 mol/L ε-ACA. Under these conditions, NB4 cells specifically bound $38.0 \pm 0.7 \times 10^6$ molecules of plasminogen per cell. For comparison, plasminogen binding was also explored on several leukemic cell lines of distinct lineages and in several types of normal peripheral blood cells. As shown in Table 1, NB4 cells bound plasminogen with a capacity at least one order of magnitude higher than the other cells analyzed.

3.2. Functional Consequences of Plasminogen Binding to NB4 Cells. Plasminogen binding to leukocytoid cells promotes plasmin formation in the presence of either tPA or uPA plasminogen activators [4–6, 22–26]. In previous kinetic studies, we have demonstrated that plasmin generation is promoted by leukocytoid cells by 60- to 30-fold when tPA or uPA, respectively, was used as plasminogen activators [5–9]. With a similar approach, we explored the promotion of plasminogen activation by NB4 cells.

NB4 cells secrete high amounts of uPA [10, 13]. uPA has a high affinity for uPA receptors, and an autocrine mechanism of saturation of uPA receptors has been previously described in several cell lines [27]. To assess whether NB4 cells could promote plasmin formation in the absence of extrinsic plasminogen activators, washed cells were incubated with plasminogen and the rate of plasminogen activation was measured as previously described [5, 6]. Under this conditions, NB4 cells generated 0.3 ± 0.01 pmol/L of plasmin · sec^{-1}, whereas 0.07 ± 0.02 and 0.06 ± 0.01 pmol/L of plasmin · sec^{-1} were generated by U937 cells or peripheral blood neutrophils, respectively. These data suggested that a significant fraction of uPA secreted by NB4 cells was bound to their cell surface and could activate plasminogen.

To better define the role of plasminogen binding sites in the promotion of plasmin formation, NB4 cells were acid treated to remove uPA from uPA receptors. Following acid treatment, <0.01 pmol/L · sec^{-1} of plasmin was generated when cells were incubated with plasminogen, indicating that the uPA was efficiently removed from cells by this treatment. Acid-treated NB4 cells were incubated with plasminogen and tPA and the rate of plasminogen activation determined. Under these conditions, plasmin generation on acid treated NB4 cells was 0.8 ± 0.04 pmol/L · sec^{-1} while U937 and peripheral blood neutrophils generated 0.3 ± 0.08 and 0.016 ± 0.04 pmol/L · sec^{-1}. of plasmin, respectively. Taken together, these data suggested that the high number of plasminogen binding sites detected on NB4 cells was paralleled by a high capacity to promote plasminogen activation on their cell surface.

3.3. Detection of Plasminogen Bound to the Cell Surface of NB4 Cells. To explore whether plasminogen could be detected on the cell surface of NB4 cells, we used an antiplasminogen monoclonal antibody (mAb49) that recognizes receptor-induced binding sites (RIBSs) in plasminogen and, therefore, preferentially react with cell-associated plasminogen in the presence of soluble plasminogen [16]. Cells were washed and incubated with plasminogen (10 μmol/L) for 1 hr at 37°C. Then, mAb49 was added to the system and processed for FACS analyses as described in Section 2. As depicted in Figure 1, mAb49 detected plasminogen bound to these cells. The mean fluorescence intensity was 2.2-fold higher in the system containing added plasminogen (5.5 units) than in the negative control without plasminogen (2.5 units).

To assess whether plasminogen binding changes induced by ATRA treatment of NB4 cells [12, 29] could also be detected by mAb49, these cells were treated for 48 hour with 5 μM ATRA. The positive FACS signal observed with untreated NB4 cells, preincubated with plasminogen, was markedly decreased after treatment of NB4 cells with ATRA for 48 hr (Figure 2(a)). For comparison, we explored with a similar approach blast cells from a patient with APL with a large proportion of blast cells (80%), both prior to and after ATRA treatment *in vivo*. A strong FACS signal with mAb49 was detected prior to ATRA treatment compared with the isotype control (Figure 2(b)). After both a 4-day and a 5-day of treatment with ATRA, blast cells exhibited a

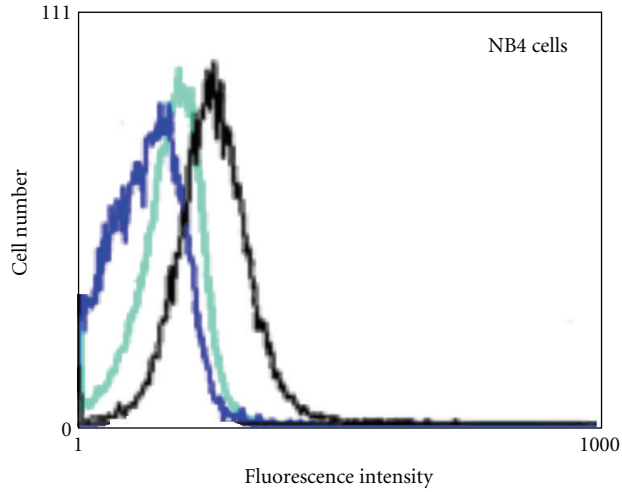

Figure 1: Detection of plasminogen bound to the surface of NB4 cells. Cells (5×10^5) were washed with PBS containing 1% BSA and 0.1% sodium azide (PBA) and incubated with 10 μM plasminogen (black tracing) or buffer (blue tracing) for 1 hour at 37°C, washed again and incubated with PBA containing 10% heat-inactivated normal rabbit serum for 10 minutes a room temperature. As an additional control, NB4 cells were treated with carboxypeptidase B (200 U/mL) before adding plasminogen (teal tracing). After incubation, supernatants were removed by centrifugation, incubated with mAb49 (130 nM) for 30 minutes a 4°C, washed and then stained with FITC-goat anti-mouse IgG, which was detected by FACS analyses. This research was originally published in [28].

markedly decreased FACS signal, compared with untreated cells (Figure 2(b)). As an additional control, FACS analysis of M1 blast cells (that have not been reported to bind plasminogen) with mAb49 did not show a positive signal compared with isotype control (Figure 2(c)).

Taken together, these results demonstrate that mAb49 can be used to monitor modulation of plasminogen to NB4 cells following ATRA treatment. Changes induced by ATRA are similar to the ones observed in blast cells of patients with APL.

4. Discussion

In this study, we have characterized plasminogen binding to the NB4 promyelocytic leukemia cell line by analyzing plasminogen binding to these cells in comparison to other blood cell lines or peripheral blood cells. The functional consequences of the high capacity of plasminogen binding by NB4 cells were explored in kinetic studies measuring plasmin generation. In addition, plasminogen binding to NB4 cells was also explored using an antiplasminogen monoclonal antibody that specifically recognizes plasminogen bound to cells. This antibody allows detection of downmodulation of plasminogen binding induced by ATRA treatment of these cells.

NB4 cells exhibited a high capacity for binding of plasminogen. This capacity is one order of magnitude higher than that displayed by other leukemic cells lines of distinct lineages and two order of magnitude higher than normal peripheral nucleated blood cells. Thus, the NB4 cell line has the highest capacity for plasminogen among the cell lines tested. This characteristic is also complemented with a high capacity to produce uPA and therefore NB4 cells have been used as models to study the bleeding complications of APL patients [10–15, 29–31]. The high plasminogen binding capacity of NB4 cells has been related to the overexpression of plasminogen binding molecules. Several proteins have been identified as cell surface binding molecules for plasminogen, including α-enolase, annexin II, tissue factor, and the complex S100A10-Annexin II [7–9, 12, 29–39]. Although annexin II was highly expressed by NB4 and APL blast cells antiannexin II antibodies reduce plasminogen activation mediated by these cells by 35%, ε-aminocaproic acid (EACA) gave a 71% reduction [12]. Because EACA inhibits the interaction of plasminogen with cells, this result implies that other plasminogen receptors in addition to annexin II could be important for stimulation of plasminogen activation. An antibody to S100A10 molecule fully blocks endothelial cell plasmin production [40], but no data have been generated using this antibody on NB4 or APL cells. A monoclonal antibody to α-enolase (11G1) blocks the cell surface promotion of plasminogen activation in a wide variety of leukemic cell lines and abrogates NB4 mediated plasminogen activation by 70–80% [41]. Thus, in addition to the Annexin II-S100A10 complex, α-enolase also mediates plasminogen binding to NB4 cells. Tissue factor is also a plasminogen-binding molecule [39] but very high concentrations of soluble TF are required to reduce plasminogen binding or cell-dependent promotion of plasminogen activation. Thus, the physiological role of TF in APL blast cells as a plasminogen binding molecule should be further studied.

NB4 cells secrete high amounts of uPA, and therefore we explore the capacity of these cells to promote plasmin generation in the absence of added plasminogen activators. Under these conditions, NB4 cells generated plasmin with a 4-5-fold higher efficiency that U937 cells or peripheral blood neutrophils. When tPA was added to acid-treated cells, promotion of plasmin generation by NB4 cells was 20–26-fold greater than by U937 cells or neutrophils, respectively. These data suggest a parallelism between plasminogen binding capacity and promotion of plasmin formation on NB4 cell surfaces.

In flow cytometric and radioimmunometric studies, we have previously demonstrated that a fraction of blood plasminogen is bound to surfaces of peripheral nucleated blood cells and platelets. In these studies, plasminogen bound to cells was detected using a monoclonal antibody to plasminogen which preferentially reacts with plasminogen bound to cell surfaces, suggesting that plasminogen binding to cells induces a conformational change in plasminogen and that latent epitopes in soluble plasminogen become available when plasminogen is bound to cells. These antibodies detect receptor-induced binding sites (RIBS) in plasminogen induced by its interaction with cells. Thus, they have been named RIBS antibodies (see details in [16]). A practical

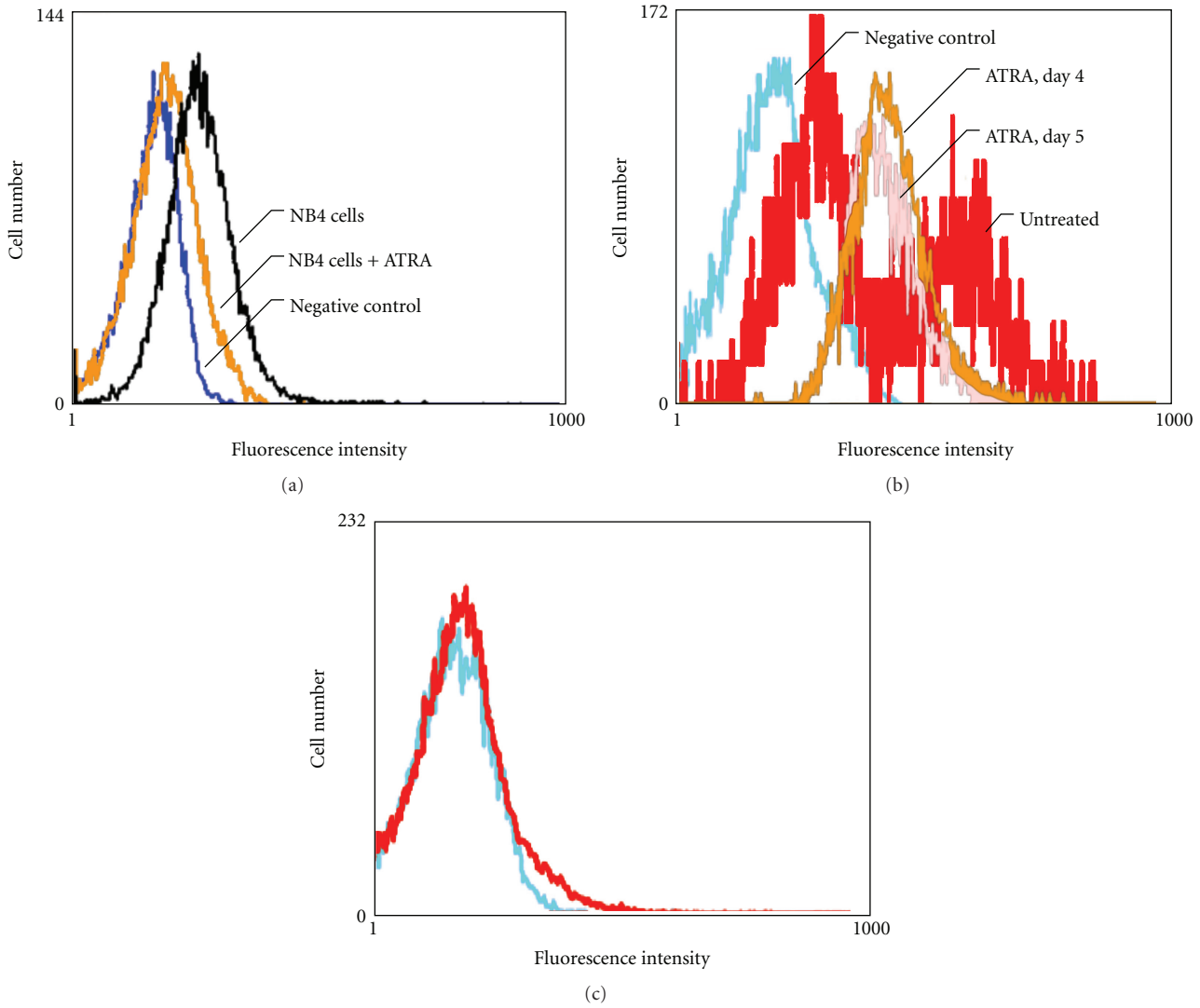

Figure 2: *In vitro* and *in vivo* ATRA modulation of plasminogen binding to NB4 cells and APL blast cells. (a) NB4 cells were incubated with 1 μM all-*trans* retinoic acid (ATRA) for 48 hours, washed and preincubated with plasminogen (10 μM) followed by FACS analyses with mAb49 (orange tracing). FACS analyses with mAb49 of untreated NB4 cells preincubated with either plasminogen (black tracing) or buffer (blue tracing) detected by antiplasminogen mAb49. (b) Plasminogen bound to blast cells from a patient with APL (CD33+; HLDR Negative) was monitorized in whole blood using mAb49. Analyses were performed at day 0 of ATRA treatment (red tracing) and after ATRA treatment for either 4 days (orange tracing) or 5 days (pink tracing). As a negative control FACS analysis with an isotype control antibody (turquoise tracing). (c) FACS analysis using mAb49 (red tracing) or isotype control (turquoise tracing) of blood from a patient with an M1 leukemia. This research was originally published in [28].

application of the use of antiplasminogen RIBS antibody has been explored on acute promyelocytic leukemia (APL) blast cells. This antibody gives a clear positive signal in FACS analyses and can be used to explore changes in the amount of plasminogen bound to blast cells in whole blood during all-*trans* retinoid acid (ATRA) treatment of APL patients [41]. Similar results were also obtained here with NB4 cells. Plasminogen was detected on NB4 cells incubated with plasminogen, whereas culture of NB4 cells with ATRA for 48 hours markedly reduced the antiplasminogen RIBS signal [41]. Again, these data reinforce the similarity between APL blast cells and the NB4 cell line. In recent studies, ATRA treatment of NB4 cells induces a downregulation of the plasminogen-binding molecule S100A10 that is paralleled by reduction in fibrinolytic activity [29]. In addition, depletion of S100A10 by RNA interference abrogates cell-dependent fibrinolytic activity in NB4 cells. Thus, these cells constitute an excellent model to explore the modulation of plasminogen binding to cells using mAb49.

5. Conclusions

Taken together, these data suggest that the NB4 cell line constitutes a unique cell model for plasmin generation on cell surfaces. The individual contribution of molecules that bind plasminogen on these cells should be explored in future

studies. In addition, the downregulation of plasminogen receptors induced by ATRA treatment of NB4 cells offers an exciting model to study the modulation of these receptors and their functional consequences.

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