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Increased expression of protease-activated receptor 1 (PAR-1) in human leukemias

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ABSTRACT

Protease-activated receptor 1 (PAR-1) is a G-protein-coupled receptor that is overexpressed in solid tumors, being associated with several pro-tumoral responses including primary growth, invasion, metastasis and angiogenesis. Expression of PAR-1 in human leukemic cell lines is reported but the status of its expression in human leukemic patients is currently unknown. In this study we evaluated the expression pattern of PAR-1 in patients with the four main types of leukemia – chronic lymphocytic leukemia subtype B (B-CLL), acute lymphoblastic leukemia subtype B (B-ALL), acute myeloid leukemia (AML) and chronic myeloid leukemia (CML). Flow cytometry analyses show that lymphocytes from B-CLL patients express this receptor at similar levels to healthy individuals. On the other hand, it was observed a significant increase in PAR-1 expression in B-ALL lymphocytes as compared to B-CLL and healthy donors. Flow cytometric and real-time PCR demonstrated a significant increase in PAR-1 expression in granulocytes from CML patients in blast phase (CML-BP) but not in chronic phase (CML-CP) as compared to healthy donors. Finally, a significant increase in PAR-1 expression has been also observed in blasts from AML (subtypes M4 and M5) patients, as compared to monocytes or granulocytes from healthy donors. We conclude that PAR-1 might play an important biological role in aggressive leukemias and might offer additional strategies for the development of new therapies.

Introduction

Protease-activated receptors (PAR) comprise a family of transmembrane G-coupled receptors (PAR-1, PAR-2, PAR-3 and PAR-4) that are uniquely activated by proteolytic cleavage of their extracellular portion. This cleavage "unmasks" a new N-terminus, which serves as a "tethered ligand" that binds to the second extracellular domain of the protein, resulting in a variety of cellular responses [1]. PAR-1, the prototypic receptor of the family, is activated by thrombin, as well as other proteases, being associated with several physiological and pathological processes [2].

Physiologically, PAR-1 is expressed by different tissues including vascular cells, neurons, fibroblasts, epithelial cells and others [2]. On the other hand, PAR-1 has been recognized as an oncogene, promoting transformation in NIH 3T3 cells [3]. PAR-1 has been shown to be overexpressed in various human cancers types including breast [4], melanoma [5,6], colon [7], prostate [8], ovarian [9], esophagus [10] and others. Moreover, studies employing cultured cells have demonstrated strong correlation between PAR-1 expression and aggressive behavior [4,11]. Thus, PAR-1 has been associated with several pro-tumoral responses in solid tumors including primary growth, invasion, metastasis and angiogenesis [4,8,11–14].

Previous studies employing human leukemic cell lines have demonstrated expression of PAR-1. Activation of PAR-1 elicits cell signaling responses which have been associated with increased production of interleukin 2 in Jurkat T cells [15]. In addition, PAR-1 is found in HL-60 cells [16] and its activation stimulates proliferation and decreases idarubicin-induced cell death in vitro [17]. Based on these data authors suggested that PAR-1 could play a role in the leukemic process. However the status of PAR-1 expression in human leukemic patients has not been fully evaluated.

The aim of this study was to evaluate the expression pattern of PAR-1 receptor in patients with the four main types of leukemia – chronic lymphocytic leukemia (CLL), acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML) and chronic myeloid leukemia (CML). Our data demonstrate for the first time that PAR-1 is significantly elevated in more aggressive leukemias including blast phase of CML, AML subtypes M4/M5 and ALL subtype B, in contrast to chronic phase in CML and CLL subtype B. Therefore, this protein might

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play an important biological role in aggressive leukemias and might offer additional strategies for the development of new therapies.

Materials and methods

Patient samples

To assess the protein expression pattern of PAR-1, we prospectively analyzed 61 peripheral blood samples (Table 1) of 7 patients with B-chronic lymphocytic leukemia (B-CLL), 11 with B-acute lymphoblastic leukemia (B-ALL), 10 with acute myeloid leukemia (AML) subtype M3, 16 with AML subtypes M4 and M5, 6 with chronic myeloid leukemia (CML) in chronic phase and 7 with CML in blast phase. Patients were from Instituto Estadual de Hematologia Arthur de Siqueira Cavalcanti (HEMORIO), Rio de Janeiro, Brazil. The median age was 35 years (range 3–82 years). Diagnosis followed the criteria proposed by the FAB classification [18]. Patients with B-ALL were stratified into high and low risk according to the following parameters: presence or absence of tumor mass and visceromegalies, leukocyte number, measurement of DHL and response to treatment.

For analysis of mRNA, 32 samples from patients with CML (23 in chronic phase and 9 in blast crisis) from Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto,Universidade de São Paulo, were included in this study. Peripheral blood samples from healthy donors were used as control for analysis of PAR-1 expression in lymphocytes, monocytes and granulocytes. This study was approved by the Ethics Committee at HEMORIO and USP and an informed consent was obtained from each patient.

Flow cytometry analysis

Expression of PAR-1 was evaluated by flow cytometry employing whole peripheral blood collected in EDTA tubes from healthy donors and leukemic patients. Cell concentration was adjusted to 1×10^6 cells/mL and incubated with 1 mL of phosphate buffered saline (PBS) containing 2% fetal calf serum for 15 min at room temperature in order to prevent nonspecific antigen-antibody complexes. Samples were then centrifuged at 2000 rpm for 5 min and the supernatant discarded. Cells were further incubated for 30 min, at 4 °C in dark, with phycoerythrin-labeled murine monoclonal antibodies against human PAR-1 (sc-13503, Santa Cruz Biotechnology Inc, USA) or isotype controls (normal mouse IgG1, Santa Cruz Biotechnology Inc., USA). After washing to remove unbound antibody, red cells were removed by incubation for 10 min with 2 mL of lysis solution (FACSLysing, ca349202, BD Biosciences, San Jose, CA) and centrifuged for 5 min at 2000 rpm. Cells were washed again and analyzed using a FACScalibur (Becton-Dickinson, USA). The mean fluorescence intensity (MFI) of 10,000 events was determined for each sample and data further analyzed using the CellQuest software. Patients evaluated in this study were classified according to their immunophenotypic profile, based on the presence or absence of specific cell surface markers for each type of leukemia. Based on the expression of these cell markers, we identified the population of mature neoplastic cells from chronic leukemias or the population of blast cells from acute leukemias. Samples were further gated for analysis of PAR-1 expression. Cell surface markers for mature cells along with analysis of cell size and citoplasmatic granularity have been used to generate gates to evaluate lymphocytes, monocytes and granulocytes from peripheral blood collected from healthy donors.

RNA isolation and reverse transcriptase real-time PCR

Blood samples were collected in EDTA from healthy donors and from patients diagnosed with CML-CP or CML-BP. Peripheral blood mononuclear cells (PBMC) were further isolated by Ficoll-Histopaque® density gradient centrifugation (Sigma-Aldrich Co., USA). Isolated cells were washed twice in PBS and total RNA was extracted using TRIZOL® reagent (Invitrogen, USA) following the manufacturer's instructions. After cDNA synthesis using Superscript III reverse transcriptase (Invitrogen), mRNA levels were determined by quantitative polymerase chain reaction

Table 1
Characteristics of the patients studied.

Sample	Age	Gender	Diagnosis	Circulating blasts in peripheral blood (%)	WBC count ($\times10^9$/L)	Flow cytometric analysis of PAR-1 (MFI)
1	64	F	B-CLL	0	84.4	0.98
2	73	F	B-CLL	0	34.4	1.56
3	77	F	B-CLL	0	126.0	2.04
4	60	F	B-CLL	0	32.6	2.09
5	68	M	B-CLL	0	201.0	2.31
6	66	F	B-CLL	0	58.2	2.50
7	66	M	B-CLL	0	66.7	2.85
8	6	M	B-ALL	68	26.6	2.03
9	32	M	B-ALL	71	44.5	2.10
10	37	F	B-ALL	96	4.2	2.12
11	15	M	B-ALL	82	336.0	2.53
12	7	F	B-ALL	52	2.0	2.78
13	17	F	B-ALL	51	ND	5.6
14	9	M	B-ALL	66	3.2	5.68
15	43	F	B-ALL	57	13.5	6.48
16	62	M	B-ALL	47	10.7	9.83
17	18	M	B-ALL	64	11.0	10.36
18	67	M	B-ALL	94	11.8	11.77
19	27	M	AML-M3	71	2.6	1.38
20	15	F	AML-M3	97	9.1	1.69
21	21	M	AML-M3	86	169.0	2.10
22	32	F	AML-M3	9	1.6	2.43
23	41	F	AML-M3	67	15.1	2.52
24	40	F	AML-M3	46	0.7	2.59
25	32	M	AML-M3	44	1.7	2.70
26	39	M	AML-M3	81	48.3	6.84
27	23	M	AML-M3	100	134.0	8.53
28	3	M	AML-M3	90	63.0	9.70
29	16	M	AML-M4/M5	28	42.0	2.56
30	58	F	AML-M4/M5	65	95.4	3.92
31	18	M	AML-M4/M5	57	100.0	4.70
32	41	F	AML-M4/M5	61	21.5	5.23
33	24	F	AML-M4/M5	94	28.3	5.62
34	32	M	AML-M4/M5	50	28.3	6.05
35	54	F	AML-M4/M5	60	29.0	8.15
36	21	F	AML-M4/M5	22	2.8	8.67
37	7	M	AML-M4/M5	5	20.2	8.75
38	82	F	AML-M4/M5	9	50.0	8.79
39	66	F	AML-M4/M5	22	2.3	8.85
40	46	M	AML-M4/M5	53	200.7	10.26
41	69	F	AML-M4/M5	13	76.6	17.06
42	43	F	AML-M4/M5	74	42.2	20.99
43	39	M	AML-M4/M5	37	91.0	25.71
44	22	F	AML-M4/M5	53	4.8	26.52
45	36	F	CML-CP	1	64.7	0.68
46	51	F	CML-CP	3	127.0	1.00
47	37	M	CML-CP	0	102.0	1.01
48	26	F	CML-CP	0	186.0	1.01
49	53	M	CML-CP	5	175.0	1.04
50	19	M	CML-CP	16	420.0	1.05
51	26	F	CML-CP	7	242.0	1.06
52	35	M	CML-CP	2	33.9	1.10
53	37	F	CML-CP	5	434.7	1.11
54	47	M	CML-CP	0	150.0	1.35
55	11	M	CML-BP	100	240.0	0.96
56	25	F	CML-BP	35	32.1	4.16
57	34	M	CML-BP	40	16.7	5.48
58	34	F	CML-BP	20	128.0	6.21
59	29	F	CML-BP	75.5	4.0	10.84
60	35	F	CML-BP	38	15.7	22.04
61	46	M	CML-BP	60	11.7	34.65

F = female; M = male; MI = Mean fluorescence intensity; B-CLL = B-chronic lymphocytic leukemia; B-ALL = B-acute lymphoblastic leukemia; AML-M3 = acute myeloid leukemia subtype M3; AML-M4/M5 = acute myeloid leukemia subtypes M4 and M5; CML-CP = chronic myeloid leukemia in chronic phase; CML-BP = chronic myeloid leukemia in blast phase; ND = non-determined.

(q-PCR) on an ABI PRISM 7500 Real Time PCR System (Applied Biosystems) using *Power* SYBR® Green PCR Master Mix (Applied Biosystems). The reaction conditions were: 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s (denaturation) and 60 °C for 1 min and the melt curve protocol began immediately after amplification. Lack of variation in PCR products and the absence of primer dimmers were ascertained from the melt curve profile of the PCR products. β-actin was used as endogenous control. Primers used were: PAR-1 (F: 5′-CAGGCACTACAAATACTGTGG-3′, R: 5′-TGTAGACTTGATT-GACGGGTT-3′) and β-actin (F: 5′-CCAGATCATGTTTGAGACCTT-3′, R: 5′-CGGAGTCATCACGATGCCAG-3′).

Statistical analysis

Results were analyzed by unpaired *t* test using Prism 4™ of Graphpad software. Results were expressed as mean ± standard deviation. Data were considered statistically significant for $p<0.05$.

Results

Expression of PAR-1 in B-CLL and B-ALL

Expression of PAR-1 has been commonly associated with a more aggressive behavior in solid tumors. In this context we first analyzed PAR-1 expression in lymphocytes from patients diagnosed with B-CLL, which is considered a non-aggressive hematological disease [19], as compared to B-ALL, which shows a more aggressive clinical behavior [20]. As control, we analyzed the expression pattern of PAR-1 in lymphocytes from healthy donors. Flow cytometry analyses show that lymphocytes from B-CLL patients express this receptor at similar levels to healthy individuals (MFI = 2.0 ± 0.2 in B-CLL vs MFI = 1.6 ± 0.1 in healthy donors). On the other hand, it was observed a significant increase in PAR-1 expression in B-ALL lymphocytes (MFI = 5.6 ± 1.1) as compared to B-CLL and healthy donors (Fig. 1). However, this observation is clearly heterogeneous, since some patients displayed a high expression pattern of PAR-1 (MFI > 5.0) while others exhibited expression levels that are similar to those observed in lymphocytes from B-CLL and healthy individuals (see Table 1).

PAR-1 expression in CML

CML is a myeloproliferative disease which presents an indolent, non-aggressive chronic phase (CML-CP) and an aggressive, blast (acute) phase (CML-BP) [21]. Given this, we performed a comparative analysis of PAR-1 expression in mature neoplastic granulocytic cells (CML-CP) and blast cells (CML-BP) from CML patients (Fig. 2). As control, we analyzed PAR-1 expression in granulocytes from healthy donors. Interestingly, it was observed a statistically significant decrease in the expression of PAR-1 in granulocytes from CML-CP patients (MFI = 1.0 ± 0.05) as compared to healthy donors (MFI = 2.3 ± 0.3). In contrast, a significant increase in PAR-1 expression was detected in the granulocytes of CML-BP patients (MFI = 12.0 ± 4.6). As seen in B-ALL patients, PAR-1 expression levels were highly heterogeneous in CML-BP, with MFI values ranging from 0.96 to 34.65 (see Table 1).

We further analyzed PAR-1 expression by quantitative real-time PCR, by employing a collection of mRNA from 32 patients diagnosed with CML. Differently from protein expression data, Fig. 3 shows that PAR-1 mRNA levels in CML-CP cells do not differ from that observed in healthy donors. Comparison between CML-BP and CML-CP showed a significant, although heterogeneous, increase in PAR-1 mRNA levels thus confirming results obtained by flow cytometry.

Expression of PAR-1 receptor in AML

In order to evaluate PAR-1 expression in AML, we further analyzed samples from patients diagnosed with AML subtype M3. Analysis of PAR-1 expression in promyeloblasts from AML-M3 patients was compared to receptor expression on granulocytes from healthy individuals. Fig. 4A shows that PAR-1 expression in AML-M3 patients (MFI = 4.0 ± 1.0) showed no statistical difference in relation to healthy individuals (MIF = 2.3 ± 0.3). It is important to note, however, that three patients showed high PAR-1 expression levels (Table 1).

Acute myelomonocytic leukemia comprises subtypes M4 and M5 in which AML-M4 is characterized by the presence of 20–80% of blast cells in the bone marrow monocytic component while AML-M5 exhibits 80% or more of non-erythroid cells in bone marrow, i.e., monoblasts, promonocytes or monocytes [18]. Therefore, analysis of PAR-1 expression was performed in patients with AML-M4/M5 as a single group. Results were compared to PAR-1 expression levels in

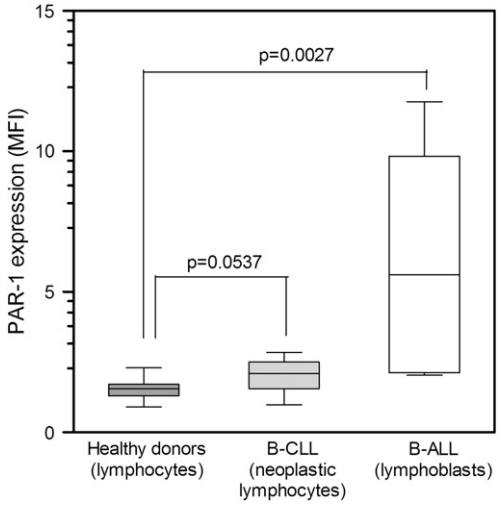

Fig. 1. Flow cytometric analysis of PAR-1 in B-CLL and B-ALL. Peripheral blood samples from healthy donors ($n = 10$), B-CLL ($n = 7$) and B-ALL patients ($n = 11$) were labeled with anti-PAR-1 antibodies and analyzed by flow cytometry as described in Materials and Methods. Statistical significance was assessed by unpaired *t* test.

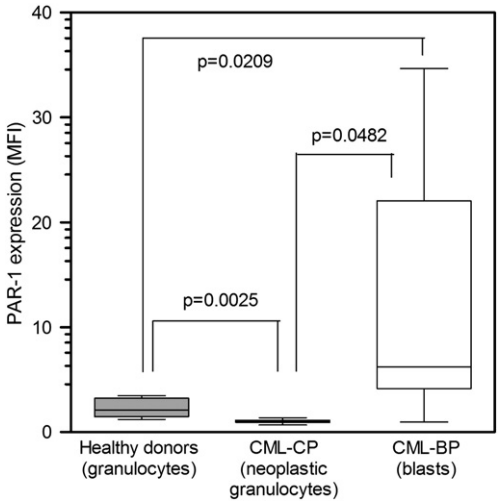

Fig. 2. Flow cytometric analysis of PAR-1 in CML. Peripheral blood samples from healthy donors ($n = 10$), CML-CP ($n = 6$) and CML-BP patients ($n = 7$) were labeled with anti-PAR-1 antibodies and analyzed by flow cytometry as described in Materials and Methods. Statistical significance was assessed by unpaired *t* test.

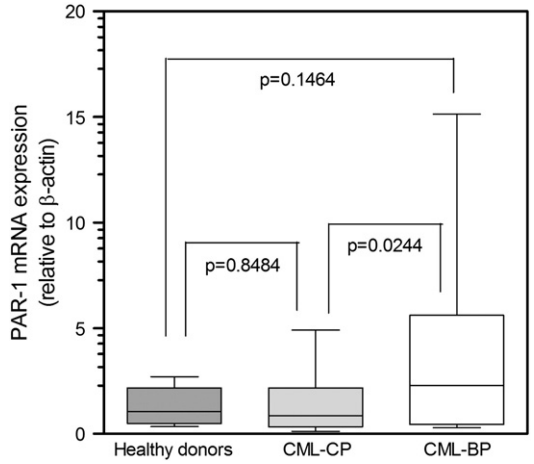

Fig. 3. Analysis of PAR-1 expression in CML by q-PCR. PAR-1 gene expression was evaluated in samples from healthy donors ($n=8$), CML-CP ($n=23$) and CML-BP patients ($n=9$) by q-PCR as described in Materials and Methods. Results were normalized to β-actin expression. Statistical significance was assessed by unpaired t test.

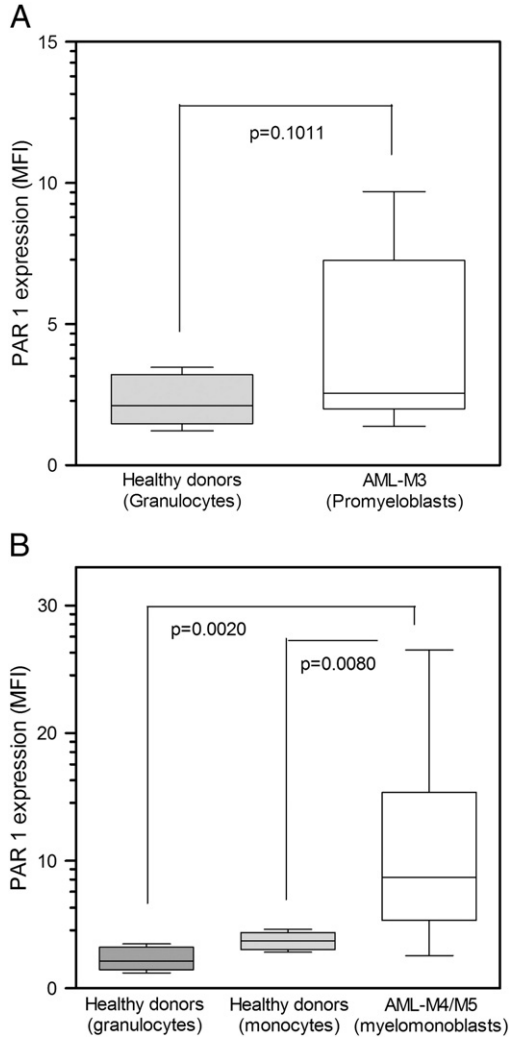

Fig. 4. Analysis of PAR-1 expression in AML. (A) Peripheral blood from healthy donors ($n=10$) or samples from AML-M3 patients ($n=10$) were probed with anti-PAR-1 antibodies and analyzed by flow cytometry as described in the Materials and Methods section. (B) Peripheral blood from healthy donors ($n=10$) or samples from AML-M4/M5 patients ($n=16$) were labeled with mAbs and analyzed by flow cytometry for PAR-1 expression. Statistical significance was assessed by unpaired t test.

granulocytes and monocytes from healthy individuals. Fig. 4B shows that patients with AML-M4/M5 display an increased expression of PAR-1 (MFI $=10.7\pm1.9$) as compared, respectively, to monocytes (MFI $=3.7\pm0.2$) or granulocytes (MFI $=2.3\pm0.3$) from healthy individuals. Most of the patients (10 out of 17) showed MFI values above 8.0 (Table 1).

Discussion

Several lines of evidence suggest that the thrombin receptor, PAR-1, plays a significant role in tumor biology. In fact, PAR-1 mediates a number of pro-tumoral responses being frequently overexpressed in solid tumors [4–10]. In the present study, we attempted to evaluate the expression pattern of PAR-1 in the main types of human leukemia. Our results demonstrate for the first time that PAR-1 is significantly elevated in more aggressive leukemias, including blast phase of CML, AML subtypes M4/M5 and ALL subtype B, in contrast with chronic phase in CML and CLL subtype B. These observations are in good agreement with data reported by Depasquale and Thompson [6] which demonstrated that PAR-1 expression is a negative prognostic factor in melanomas and strongly correlates with tumor stage.

Patients with B-CLL, in most cases, have an indolent clinical course which is asymptomatic and requires no treatment [19]. On the other hand, B-ALL is believed to derive from blockade in maturation of bone marrow lymphoid progenitors leading to bone marrow infiltration, occurrence of various cytopenias in peripheral blood and appearance of blast cells with high proliferative ability. Therefore, patients with B-ALL show a more aggressive clinical behavior than patients with B-CLL [20]. In this study we observed that patients with B-CLL showed similar PAR-1 expression levels in comparison to lymphocytes from healthy individuals. On the other hand, patients with B-ALL exhibited, on average, a significant increase in the expression of this receptor when compared to normal lymphocytes. Interestingly, patients classified as at high risk showed the highest PAR-1 levels among B-ALL patients.

CML is a myeloproliferative disease which is characterized by the presence of the fusion gene BCR-ABL, an oncoprotein generated by reciprocal translocation between chromosomes 9 and 22, $t(9;22)$ [21]. In this study, flow cytometric analyses demonstrated that CML patients in the chronic phase (CML-CP) exhibited a significant decrease in PAR-1 when compared to granulocytes from healthy individuals. Since PAR-1 has been implicated in inflammatory responses as well as in innate and adaptive immunity [22], it is possible that down-regulation of this receptor may contribute for reduced function of these cells and increased susceptibility to recurrent infections in CML. Progression of CML-CP to CML-BP is not fully understood. In fact it is believed that BCR-ABL in cooperation with other factors may account for accelerated leukemogenesis and drug resistance in the acute phase [21]. In this study, we identified an increased PAR-1 expression in blasts from CML-BP patients. However, it is clear that a more extensive analysis is needed to determine the biological significance of these results.

Acute myelomonocytic leukemia, which comprises subtypes M4 and M5, are highly aggressive and have a median survival time of only 12 months [23]. Samples from AML-M4/M5 patients that were analyzed in this study exhibited high levels of PAR-1 receptor when compared to monocytes and granulocytes from healthy donors. In contrast, patients with AML-M3 showed PAR-1 expression levels that were similar to those found in granulocytes from healthy donors. However, 3 out of 10 patients exhibited high levels of this receptor. Interestingly, these patients were non-responsive to treatment with trans-retinoic acid (ATRA), a potent inducer of cell differentiation process [24], and further died as consequence of hemorrhagic disturbances. In fact, patients with AML-M3 are at increased thrombotic risk and hemorrhagic complications following disseminated intravascular coagulation (DIC) [25]. These

serious complications have been attributed to the aberrant expression of the clotting initiator protein, tissue factor (TF), in blast cells [26,27]. Treatment with ATRA down-regulates TF expression and reduces activation of blood coagulation in AML-M3 patients [28,29]. More recently, Barbarroja and co-workers [30] suggested that TF is involved in the activation of multiple signaling pathways in leukemic cells. At this point, patients that are non-responsive to ATRA may exhibit an increased TF-mediated thrombin generation and augmented activation of PAR-1 in leukemic cells which may contribute to disease progression. In this regard, it is proposed that TF inhibitors may reduce thrombin generation and exert antitumor effects, at least in part, by indirectly decreasing PAR-1 signaling [31].

In summary, our study demonstrates for the first time that PAR-1 expression is significantly elevated in more aggressive leukemias including blast phase of CML, AML subtypes M4/M5 and ALL subtype B, in contrast to chronic phase in CML and CLL subtype B. Therefore, this protein might play an important biological role in aggressive hematologic malignancies and might offer additional strategies for the development of new therapies.

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