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Mechanism of paroxysmal nocturnal hemoglobinuria clonal dominance: possible roles of different apoptosis and CD8+ lymphocytes in the selection of paroxysmal nocturnal hemoglobinuria clones

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BACKGROUND AND OBJECTIVES: Paroxysmal nocturnal hemoglobinuria (PNH), a clonal hematopoietic stem cell disorder, manifests when the PNH clone populates in the hematopoietic compartment. We explored the roles of different apoptosis of GPI+ and GPI- (glycosylphosphatidylinositol) cells and CD8+ lymphocytes in a selection of PNH clones.

PATIENTS AND METHODS: Granulocytes from PNH patients and normal controls were subjected to an apoptosis assay using annexin V. Hematopoietic cell in semisolid media were cultured with or without CD8+ lymphocytes.

RESULTS: In PNH, CD59+ granulocytes exhibited more apoptosis than their CD59- counterparts, after 0 or 4 hours in liquid growth culture system (mean [standard error of mean]: 2.1 (0.5) vs 1.2 (0.2), *P*=.01 at 0 hour and 3.4 [0.7] vs 1.8 [0.3], *P*=.03 at 4 hour, respectively). The presence of mononuclear cells (MNCs) rendered a greater difference in apoptosis. The percentages of apoptotic CD59+ granulocytes measured at 4 hours with or without MNC fraction were correlated with the sizes of PNH clones (r=0.633, *P*=.011; and r=0.648, *P*=.009; respectively). The autologous CD8+ lymphocytes inhibited CFU-GM and BFU-E colony formation in PNH patients when compared with normal controls (mean [SEM] of percentages of inhibition: 61.7 (10.4) vs 11.9 (2.0), *P*=.008 for CFU-GM and 26.1 (6.9) vs 4.9 (1.0), *P*=.037 for BFU-E).

CONCLUSIONS: Increased apoptosis of GPI+ blood cells is likely to be responsible in selection and expansion of PNH clones. MNCs or possibly CD8+ lymphocytes may play a role in this phenomenon.

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired, clonal hematopoietic stem cell disorder characterized by the unique triad of complement-mediated intravascular hemolytic anemia, bone marrow failure, and thrombosis.[1] PNH originates from a somatic mutation of an X-linked gene, *PIGA*, in a hematopoietic stem cell. The *PIGA* gene encodes a protein subunit of the enzyme complex required for the initial step in the biosynthesis of glycosylphosphatidylinositol (GPI).[2] GPI is a glycolipid moiety, which attaches certain proteins to the cell membrane. Clinical PNH results from expansion of the *PIGA* mutated clone that is deficient in GPI anchored proteins.[3] Lacking the GPI-anchored proteins is the hallmark of PNH blood cells and responsible for certain clinical features of PNH. For example, intravascular hemolytic anemia occurs as a consequence of the deficiency of the GPI-anchored membrane inhibitor of reactive lysis (CD59) and decay-accelerating factor (DAF).[4]

It is unclear how the PNH clone arising from the mutated hematopoietic stem cell expands in the bone marrow. The *PIGA* mutation itself does not appear to favor growth advantage of the GPI-deficient blood cells. In chimeric, *PIGA* knocked-out mice, the relative size

of GPI-deficient clone declined during the prolonged follow-up period.[5] Moreover, very small populations of GPI-deficient blood cells containing *PIGA* mutations have been shown to be present without a tendency to expand in normal persons.[6] On the other hand, preferential PNH hematopoiesis was observed following its engraftment in severe combined immunodeficiency (SCID) mice.[7] All the above data suggest that the *PIGA* mutation alone may not be sufficient for clonal expansion of PNH-type cells but is likely to confer a selective advantage in certain conditions.

PNH is closely linked to disorders with bone marrow failure, especially aplastic anemia.[8] PNH frequently emerges during the clinical course of aplastic anemia and more than 30% of patients with PNH had a precedent history of aplastic anemia.[9] Moreover, deficient hematopoiesis is a feature of PNH as evidenced by the frequently observed leukopenia/thrombocytopenia, hypocellular marrow, low number of CD34+ and progenitor cells CFU-GM and BFU-E.[3] In addition, PNH-type (GPI-deficient) blood cells have been reported in more than 50% of patients with aplastic anemia.[10,11] Persistence and expansion of these PNH-type cells to the level of clinical PNH have been observed in a proportion of patients.[10] Taken together, all of the findings suggest that the expansion of a GPI-deficient clone is the result of (favorable) selection by a condition responsible for the underlying bone marrow failure, ie aplastic anemia.[12]

Based on the underlying immunologic mechanism of aplastic anemia, it is likely that escape from immunologic attack by T or natural killer (NK) cells is responsible for the selective survival of *PIGA* mutant hematopoietic stem cells.[13] The findings of large granular lymphocyte (LGL)-like clonal expansions and abnormal T-cell repertoire by size analysis of the complementarity-determining region 3 (CDR3) in the beta variable chain (BV) messenger RNA (mRNA) of the T-cell receptor (TCR) in PNH patients also favor the role T-cell mediated immune processes in the pathogenesis of PNH.[14,15] According to the above suggestive evidence, PNH is likely to share a common pathogenesis with aplastic anemia, which is autoimmune T-cell mediated attack of hematopoietic stem cells, with a sparing and subsequent expansion of GPI- cells. Recently, increased susceptibility to apoptosis of GPI+ cells has been shown to be responsible to preferential growth of GPI- cells in PNH patients.[16] Since apoptosis of targets may be a consequence of T-cell mediated cytotoxicity,[17] it is possible that increased apoptosis in GPI+ hematopoietic stem cells in patients with PNH is the result of T-cell mediated autoimmune selection of PNH hematopoiesis. Our study was, therefore, designed to explore the roles of different apoptosis and cytotoxic (CD8+) lymphocytes in the selection of PNH clones in patients with PNH.

PATIENTS AND METHODS

Patients and normal controls

PNH patients followed at the Division of Hematology, Department of Medicine, Faculty of Medicine, Siriraj Hospital, Mahidol University, voluntarily participated in this study. The diagnosis of PNH was confirmed by positive Ham test and/or the presence of CD59- blood cells (erythrocytes and/or granulocytes) by flow cytometric analyses. Thirty-three healthy volunteers were also included in this study as normal controls. This study was approved by the ethical committee (IRB) of the Faculty of Medicine, Siriraj Hospital, Mahidol University.

Blood samples and cell separation

Twenty mL of heparinized blood obtained from the patients and healthy volunteers after obtaining informed consent, was divided into two parts. A portion of 10 mL of the heparinized blood was separated for leukocyte fraction (from erythrocytes) by 6% dextran sedimentation. The leukocyte fraction was then subjected to centrifugation gradient through Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) to separate mononuclear cells (MNCs) from polymorphonuclear cells (PMNs).[18] After washing with phosphate buffered saline (PBS), and being suspended in Rosewell Park Memorial Institute (RPMI)-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 1% L-glutamine, 10% fetal calf serum (FCS), 50 μg/mL streptomycin, and 50 IU/mL penicillin, the leukocyte suspension was used for apoptosis analyses.

The second part of the 10 mL of heparinized blood was separated for MNC fraction by centrifugation gradient through Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). The separated MNCs were washed twice and suspended with PBS/EDTA buffer at a concentration of 107/100 μL for the uses of hematopoietic progenitor cell co-culture study and also for purification of CD8+ and CD8- cells.

Apoptosis and flow cytometry analysis

Apoptosis analysis was carried out following a number of pilot experiments to find out the optimal condition for the study. Briefly, 1×10^6 polymorphonuclear (PMN) leukocytes were cultured alone as control and co-cultured with 1×10^6 MNCs in 1 mL of RPMI-1640

supplemented with 1% L-glutamine, 10% FCS, 50 μg/mL streptomycin, and 50 IU/mL penicillin. After 0- and 4-hour cultures or co-cultures, 0.5 mL of cell suspension was incubated with 50 μl of anti-CD59 biotin conjugated, 7 μl of anti-CD45 per-CP, 2 μL of annexin-V FITC, and 100 μL of binding buffer and incubated at 2°C to 8°C for 15 minutes. Annexin-V binding in the CD59+ and CD59- cell populations were determined by three-color analysis of flow cytometry. The gated granulocytes, based on side- and forward-scatter properties and expression of CD45, were analyzed for annexin-V positivity as shown in dot plots separated in four quadrants (**Figure 1**). Apoptotic CD59+ and CD59- granulocytes are shown in upper right (UR) and lower right (LR) quadrant, while non-apoptotic CD59+ and CD59- granulocytes are shown in upper left (UL) and lower left (LL) quadrant, respectively. The proportion of apoptotic CD59+ or CD59- granulocytes was expressed in percentage as the number of apoptotic granulocytes (UR or LR) divided by the total number of each granulocyte population (UR+UL or LR+LL).

Purification of CD8- and CD8+ cells

Purification of CD8- cells was done by indirect immune-magnetic activated cell sorter. Briefly, 107 of MNCs in 100 μL PBS/EDTA were incubated with 70 μl of anti-CD8 PE antibody for 15 minutes at room temperature (RT). The stained cells were washed with 50 mL of the buffer with centrifugation at 2000 rpm for 5 minutes. The cell pellet was then suspended in 80 μL of buffer and incubated with 20 μL of goat anti-mouse-IgG microbeads for 15 minutes at 2°C to 8°C. The suspension was subsequently washed with the same amount of the buffer and centrifugation. After complete discard of supernatant, the cell pellet was re-suspended in 0.5 mL of buffer. The final cell suspension was applied onto the prepared LS column (Miltenyi Biotec, Earhart Avenue Auburn, CA, USA), for magnetic separation of CD8+ and CD8- fractions according to the manufacturer's guideline.

Hematopoietic progenitor cell assay

After purification by indirect immune-magnetic activated cell sorter, 4×10^5 of CD8- cells and 1×10^5 of CD8+ cells were co-cultured in methocult (Stem Cell Technologies, Vancouver, Canada), containing 1% methylcellulose in IMDM, 30% FBS, 1% BSA, 10-4 M 2-mercaptoethanol, 2 mM L-glutamine, 50 ng/mL stem cell factor, 10 ng/mL GM-CSF, 10 ng/mL interleukin 3, and 3 U/mL erythropoietin, and plated onto 35 mm culture dishes in duplication. As a control, 4×10^5 cells from CD8- cell fraction were also cultured alone. Each culture was incubated for 14 days at 37°C in 5% CO_2 condition. Colonies consisting of at least 50 cells were enumerated and characterized, according to their morphology, by an inverted microscope.

Statistical analysis

Differences between groups and parameters were evaluated by the Mann-Whitney test and the Wilcoxon signed-rank test. The Pearson correlation was used to determine the relationship between the proportions of the apoptotic CD59 negative and positive granulocytes and hematological parameters. Statistical significance was considered when the *P* value was <.05.

RESULTS

Patient characteristics and the sizes of PNH clones

Among the 15 patients studied, there were 6 females and 9 males who ranged in age from 28 to 68 years with a median age of 39 years (**Table 1**). Ten (67%) of the patients were in the category of classic or hemolytic PNH while the other 5 (33%) had cytopenias other than anemia and were classified as PNH/aplastic anemia (PNH/AA). Six patients (40%) had a pre-existing history of aplastic anemia. Nearly all patients had mild disease and their base-lined hemoglobin concentrations; defined as the average hemoglobin concentrations of the previous three visits before studies, were 5-13 g/dL (mean and median were equal at 10.2 g/dL). All except two patients were transfusion independent. Sizes of PNH clones as estimated by the percentages of CD59- granulocytes were between 25.9% to 98.3%, the mean (standard error of mean) was 68.1 (6.6) and median 75.6.

Different apoptosis between CD59+ or CD59- granulocytes

The percentages of apoptosis of granulocytes (PMNs) in liquid growth culture without and with 1:1 mononuclear cell fraction are shown in **Table 2**. After 0 hour or 4 hours in liquid growth culture system, CD59+ granulocytes from PNH patients exhibited more apoptosis than the CD59- counterparts (mean [standard error of mean]: 2.12 [0.47] median 1.59, vs 1.20 [0.20], median 0.96, *P*=.01 at 0 hour and 3.35 [0.67], median 2.95 vs 1.82 [0.26], median 1.62, *P*=.03 at 4 hours, respectively). Co-culture of granulocytes with mononuclear cells for 4 hours seemed to render more difference in apoptosis between the CD59+ granulocytes (mean [SEM]: 3.35 [0.66] median 2.25) and the CD59- cells (mean [SEM]: 1.67 [0.29], median 1.60); *P*=.005.

Table 1. Demographic, clinical, hematologic data and sizes of PNH clones (% CD59- granulocytes).

UPN	Sex	Age (yrs)	Disease category	Hb (g/dL)	WBC ($\times 10^9$/L)	Platelet ($\times 10^9$/L)	% CD59- granulocytes
1	F	28	PNH/AA	5.0	4.8	92	98.30
2	M	38	Classic PNH	7.5	6.08	198	79.65
3	M	50	Classic PNH	8.4	5.73	49	27.07
4	F	55	Classic PNH	12.4	5.0	355	91.40
5	F	38	Classic PNH	11.0	6.67	142	75.57
6	M	68	PNH/AA	9.4	3.66	32	25.87
7	F	31	PNH/AA	12.1	3.14	71	36.74
8	F	60	Classic PNH	10.2	7.41	178	94.7
9	M	47	Classic PNH	11.1	11.64	172	65.16
10	M	39	Classic PNH	9.2	4.18	170	34.40
11	M	35	PNH/AA	10.6	2.69	43	66.40
12	F	45	Classic PNH	11.4	6.06	100	75.84
13	M	39	Classic PNH	13.1	4.22	91	97.0
14	M	34	PNH/AA	11.0	4.40	41	84.73
15	M	32	Classic PNH	10.0	6.04	230	69.06

UPN=unique patient number

Table 2. Percentages of apoptosis of CD59+ and CD59- granulocytes.

	% apoptosis of granulocytes in culture							
	Without mononuclear cells				With mononuclear cells			
Time of culture	0 hour		4 hours		0 hour		4 hours	
Cell population	CD59+	CD59-	CD59+	CD59-	CD59+	CD59-	CD59+	CD59-
Normal controls	0.74 (0.11)[d]	ND	3.24 (0.52)	ND	0.68 (0.07)[e]	ND	2.10 (0.37)[f]	ND
PNH patients	2.12 (0.47)[a,d]	1.20 (0.20)[a]	3.35 (0.67)[b]	1.82 (0.26)[b]	1.78 (0.33)[e]	1.31 (0.27)	3.35 (0.66)[c,f]	1.67 (0.29)[c]

[a] P=.01; [b] P=.03; [c] P=.005; [d] P=.002; [e] P=.002; [f] P=.02. Values are mean (standard error of mean).

When compared with normal controls, CD59+ granulocytes from PNH patients were more apoptotic than the CD59+ granulocytes from normal controls in culture without MNCs at 0-hour culture (mean [SEM]: 2.12 [0.47], median 1.59 for PNH patients vs 0.74 [0.11], median 0.47 for normal controls; P=.002). Similar findings were also observed in co-culture with MNCs at 0-hour culture (mean [SEM]: 1.78 [0.33], median 1.28 for PNH patients vs 0.68 [0.07], median 0.60 for normal controls; P=.002) or at 4-hour culture in the presence of MNCs (mean [SEM]: 3.35 [0.66], median 2.25 for PNH patients vs mean [SEM]: 2.10 [0.37], median 1.36 for normal controls; P=.02) (**Figure 2**). However, the percentages of apoptotic CD59+ granulocytes from normal controls and the patients in culture without MNCs were not different (mean [SEM]: 3.35 [0.67], median 2.95 for PNH patients vs 3.24 [0.52], median 2.44 for normal controls; P=.40).

The relationship between the percentages of apoptotic CD59+ granulocytes and hematologic parameters and sizes of PNH clones

The percentages of apoptotic CD59+ granulocytes measured at 4 hours without or with MNC fraction were correlated with the sizes of PNH clones as represented by percentages of CD59- granulocytes (r=0.648, P=.009 and r=0.633, P=.011; respectively). They also

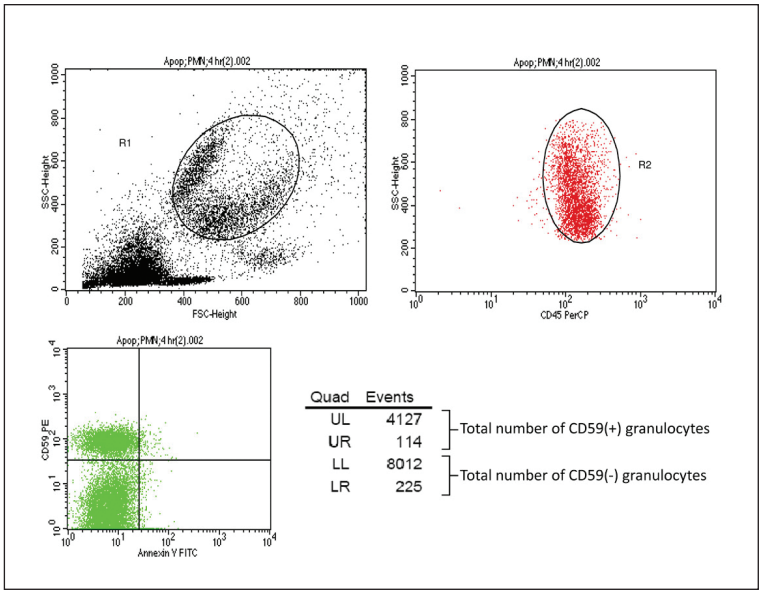

Figure 1. Flow cytometric analysis of apoptotic granulocytes as shown by dot-plot scattergram. Granulocytes were gated with forward and side scatters (FSC/SSC) (R1). Only CD45 (leukocyte antigen) positive granulocytes (R2) were further analyzed to examine expression of GPI-anchored CD59 and annexin-V binding. Lower dot-plot scattergram shows granulocytes with normal phenotype (CD59+ granulocytes) in upper left (UL) and upper right (UR) quadrants. Granulocytes with PNH phenotype (CD59- granulocytes) are shown in lower left (LL) and lower right (LR) quadrants. Granulocytes with annexin-V positive indicating cell apoptosis are shown in UR and LR quadrants; non-apoptotic granulocytes with annexin-V negative are shown in UL and LL quadrants.

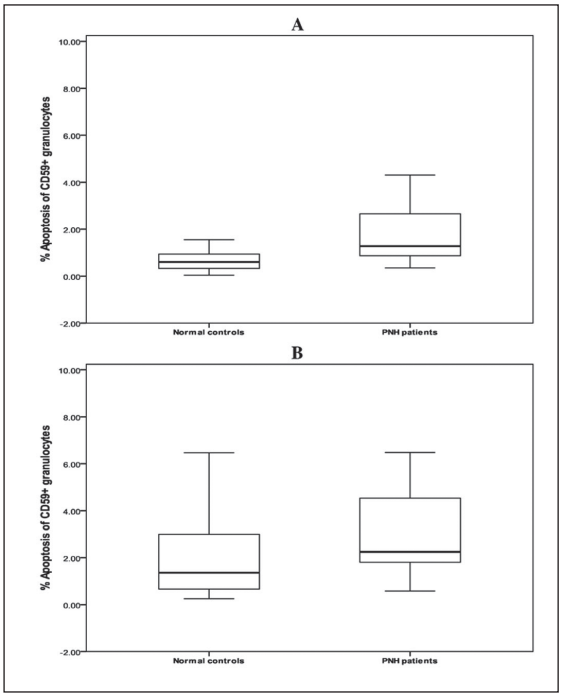

Figure 2. Comparison of percentages of apoptotic CD59+ granulocytes from normal controls and patients with PNH. More apoptotic CD59+ granulocytes were observed in the patients than in controls in both cultures with MNCs at 0 hour **(A)** (mean [SEM]: 1.78 [0.33], median 1.28 for PNH patients vs 0.68 [0.07], median 0.60 for normal controls; *P*=.002) and at 4 hours **(B)** (mean [SEM]: 3.35 [0.66], median 2.25 for PNH patients vs mean [SEM]: 2.10 [0.37], median 1.36 for normal controls; *P*=.02). The results are expressed in box plot as medians, 25th-75th, and 10th-90th percentiles.

had negative correlations with the base-lined Hb concentration of patients (r=-0.722, *P*=.002 and r=-0.649, *P*=.009; respectively) (**Figure 3**). While the percentages of apoptotic CD59+ granulocytes measured at 0 hour did not correlate with hemoglobin concentration, they had some correlation with the sizes of PNH clones (r=0.517, *P*=.049). There was no significant relationship between apoptotic CD59+ granulocytes and other hematologic parameters, such as white blood cell count or platelet count.

Hematopoietic progenitor growth with or without CD8+ lymphocytes

Hematopoietic progenitor growth with or without CD8+ lymphocytes were studied in 5 patients with PNH and 5 normal controls. In accordance with our previous study,[3] the numbers of BFU-E and CFU-GM in patients with PNH were significantly lower than those from normal controls. When compared between cultures performed with or without the CD8+ fraction, the numbers of BFU-E and CFU-GM in normal controls were not different (mean [SEM]: 220.30 [20.85] vs 234.60 [23.26], *P*=.18 for BFU-E and 15.70 [2.65] vs 16.90 [3.69], *P*=.59 for CFU-GM).

In contrast, the numbers of CFU-GM of PNH patients were lower in culture with the presence of CD8+ fraction than those in culture without CD8+ cells. (mean [SEM]: 1.50 [0.71] vs 3.10 [1.16], *P*=.04 for CFU-GM and 28.80 [8.96] vs 30.20 [10.06], *P*=.67 for BFU-E). The percentages of inhibition by CD8+ lymphocytes, as represented by the differences of the numbers of colonies in cultures with and without CD8+ cells divided by the numbers of colonies in cultures without CD8+ cells, were significantly higher for both CFU-GM and BFU-E in PNH patients as compared to normal controls (mean [SEM]: 61.69 [10.37] vs 11.93 [1.96], *P*=.008 for CFU-GM and 26.08 [6.9] vs 4.90 [1.00], *P*=.037 for BFU-E) (**Figure 4**).

DISCUSSION

It remains unclear how a GPI-deficient clone arising from a mutated hematopoietic stem cell expands in PNH. Ample evidence suggest that the selective expansion of PNH clones is not simply attributable to the *PIGA* mutations alone.[19,20] The strong association of PNH with aplastic anemia, in which immune (T cell)-mediated marrow destruction has been inferred as the underlying pathophysiology, suggests an immune mechanism of selection. However, so far there has been no in vitro experiment which directly demonstrates both the cellular and molecular mechanism of immune escape of the PNH clone.[21] Recently, there have been

studies suggesting that difference in apoptosis of GPI+ and GPI- cells may be responsible for the clonal expansion of GPI-negative (PNH) clone.[16,22]

In our study, we explored both the effect of immune selection via CD8 cells, which represented cytotoxic lymphocytes and also the role of different apoptosis in PNH clonal evolution. Using peripheral blood granulocytes as representative cells, we found that the GPI+ cells (CD59+ granulocytes) from PNH patients exhibited more apoptosis than their GPI- counterparts (**Table 2**). In the presence of mononuclear cells (for 4 hours), the difference of apoptotic tendency between the two cell populations seemed to be more. Moreover, the GPI+ granulocytes from PNH patients were more apoptotic than those from normal controls, including in the cultures with mononuclear cells (**Figure 2**). Our findings as well as those from other studies suggest that different apoptosis between GPI+ and GPI- blood cells plays a role in PNH clonal evolution.[16,22] Our findings of the relationships between the magnitude of apoptotic CD59- granulocytes with the sizes of PNH clones and the base-lined hemoglobin concentrations (**Figure 3**) also supported the significance of the difference in apoptosis of GPI+ and GPI- cells in the pathogenesis of PNH. More apoptosis of the GPI+ cells leads to PNH clonal dominance by leaving more survival of the GPI- (PNH) cells. The sizes of PNH clones also determine severity of the disease or anemia as suggested by our previous study,[23] which may explain the negative correlation between the percentages of apoptotic CD59- granulocytes and the base-lined hemoglobin concentrations of the patients.

It is debatable whether the difference in apoptotic rates of GPI+ and GPI- cells is due to the resistance of GPI- blood cells to apoptosis[24] or because the GPI+ cells are susceptible to apoptosis.[25] There have been controversial data suggesting that a mutation of *PIGA* confers resistance to apoptosis to GPI deficient blood cells leading to expansion of the PNH clone.[24-27] Our findings of higher apoptosis rates of GPI+ cells from PNH patients than those from normal controls, other than the GPI- counterparts, suggests that the non-mutated *PIGA* cells are predisposed to apoptosis leaving the GPI-deficient blood cells to be selected and subsequently expand. Our data provide additional evidences supporting the concept that PNH clonal evolution occurs as a result of increased susceptibility of GPI+ cells to apoptosis, as suggested by a study using DNA microarray.[28] It is likely that this phenomenon occurs as a result of extrinsic influence, constituted as the selective immunologic attack of GPI+ blood cells by effectors such as cytotoxic T or NK lymphocytes leading to induction of apoptosis of the target cells.[28-30] The finding of a tendency of increased apoptosis in the presence of MNCs which are a source of effector cells also favors this suggestion. Although our findings do not explain why the unmutated GPI+ cells are selectively destroyed, it is likely that the GPI-anchored proteins or the GPI itself are the prime targets of the immune recognition by the effector cells.

With hematopoietic progenitor cell assay, the numbers of BFU-E and CFU-GM in patients with PNH

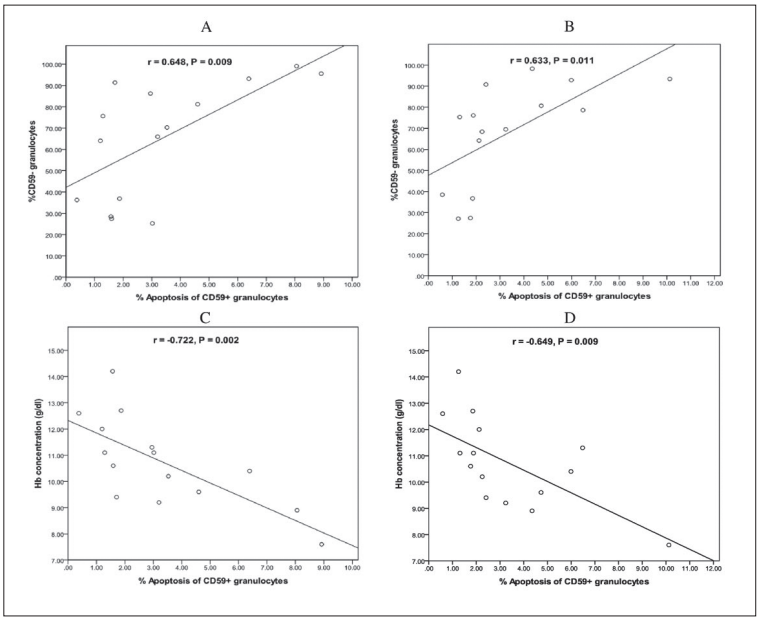

Figure 3. Scattergram to demonstrate the correlation between % apoptosis of CD59+ granulocytes and % CD59- (PNH) granulocytes in cultures at 4 hours without MNCs **(A)** (r=0.648, *P*=.009) and with MNCs **(B)** (r=0.633, *P*=.011). While, the percentages of apoptotic CD59+ granulocytes had negative correlations with Hb concentrations in cultures at 4 hours without MNCs **(C)** (r=-0.722, *P* =.002) and with MNCs **(D)** (r=0.649, *P*=.009).

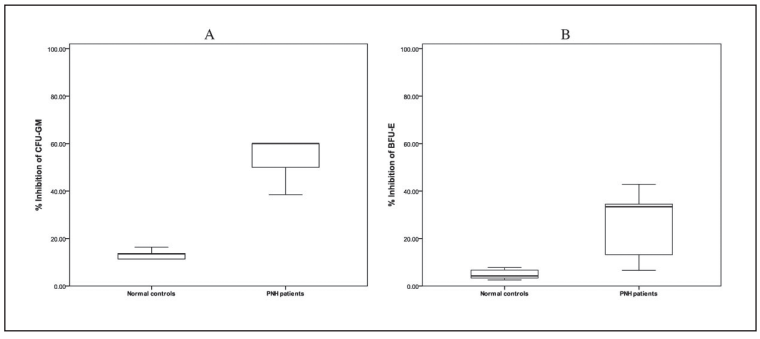

Figure 4. Effect of CD8+ lymphocytes on CFU-GM and BFU-E formations. The results are shown by box-plots of percentages of inhibition. The percentages of inhibition by CD8+ lymphocytes were significantly higher in PNH patients than normal controls for both CFU-GM **(A)** (mean [SEM]: 61.69 [10.37] vs 11.93 [1.96], *P*=.008) and BFU-E **(B)** (26.08 [6.9] vs 4.90 [1.00], *P*=.037).

were significantly lower than those from normal controls, indicating a condition of deficient hematopoiesis in patients with PNH.[3] In the patients, but not in the normal controls, the numbers of CFU-GM were significantly lower in cultures with autologous CD8+ lymphocytes than in those without these cells. Moreover, the effects (percentages) of inhibition of progenitor cell growth (both CFU-GM and BFU-E) by autologous CD8+ lymphocytes were more in the patients than in normal controls. Our findings are consistent with other studies of different approaches and suggest that cytotoxic T lymphocytes (CTLs) are responsible for the condition of deficient hematopoiesis or bone marrow failure and also play an important role in the immune selection of PNH clones in patients with PNH.[14,31,32] Our findings do not exclude the role of NK cells in immune selection of PNH blood cells as suggested by Hanaoka et al[30] since CD8+ may also be expressed on NK cells.[33] However, a study has shown that NK cells may be less efficient in targeting GPI-deficient cells.[34]

Under the process of immune selection, the CTLs are supposed to target and inhibit growth of the GPI+ hematopoietic stem/progenitor cells through effector mechanisms which may involve direct granzyme/perforin cytotoxicity, CTL-mediated secretion of inhibitory cytokines such as IFN-α and TNF-α.[35] All of these mechanisms have been shown to induce apoptosis of target cells.[17] In spite of using different approaches and targets, our findings of inhibition of progenitor cell growth by CTLs and increased apoptosis of CD59+ granulocytes convince the role of CTLs in preferential growth of GPI- hematopoietic stem cells through cytotoxicity-induced apoptosis. Elevated Fas receptor expression in GPI+ stem cells, which was found by some investigators,[25] may render more CTL-induced apoptosis of these cells and enhance more growth advantage of GPI- stem cells leading to PNH clonal dominance.

In summary, we have shown that more apoptosis of the GPI+ blood cells than their GPI- counterparts is responsible for the selection of PNH clones with subsequent clonal expansion in PNH. MNCs, especially the auto-reactive CD8+ lymphocytes, may play a role in this phenomenon by selectively suppression of non-PNH hematopoiesis, through cytotoxicity-induced apoptosis.

Acknowledgments

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