Table 3. Crude Incidence and Crude Relative Risk of Myelodysplastic Syndromes by Exposure Status in Nagasaki Atomic Bomb Survivors

Variable	Nagasaki Atomic Bomb Disease Institute Cohort						Life Span Study-Nagasaki Cohort					
	Exposure Distance (km)				Crude		Weighted Bone Marrow Dose (Gy)				Crude	
	< 1.5	1.5-2.99	≥ 3.0	Total	RR	95% CI*	≥ 1	0.005-0.999	< 0.005	Total	RR	95% CI*
Sex												
Male												
Population at risk	1,693	6,485	16,092	24,270			273	2,665	5,904	8,842		
No. of patients	12	21	34	67			3	8	10	21		
Person-years	23,071	91,880	233,191	348,144			2,959	29,789	66,102	98,850		
Crude rate†	52.0	22.9	14.6	19.2	1.3	1.0 to 1.9	101.4	26.9	15.1	21.2	1.4	0.8 to 2.5
Female												
Population at risk	2,258	10,663	26,835	39,756			351	4,201	8,851	13,403		
No. of patients	13	23	48	84			5	7	14	26		
Person-years	34,946	158,144	405,980	599,071			4,480	52,926	114,363	171,769		
Crude rate†	37.2	14.5	11.8	14.0	Ref		111.6	13.2	12.2	15.1	Ref	
Age at exposure, years												
0-9												
Population at risk	615	4,770	13,730	19,115			161	2,464	5,064	7,689		
No. of patients	6	9	13	28			3	6	3	12		
Person-years	9,756	77,132	225,071	311,960			1,750	29,274	60,572	91,596		
Crude rate†	61.5	11.7	5.8	9.0	Ref		171.4	20.5	5.0	13.1	Ref	
10-19												
Population at risk	1,950	5,620	13,611	21,181			280	2,256	4,841	7,377		
No. of patients	13	16	29	58			2	5	8	15		
Person-years	31,325	91,011	225,009	347,346			3,532	29,182	63,714	96,428		
Crude rate†	41.5	17.6	12.9	16.7	1.9	1.2 to 3.0	56.6	17.1	12.6	15.6	1.2	0.6 to 2.5
≥ 20												
Population at risk	1,386	6,758	15,586	23,730			183	2,146	4,850	7,179		
No. of patients	6	19	40	65			1	11	8	20		
Person-years	16,937	81,882	189,091	287,909			2,157	24,259	56,179	82,595		
Crude rate†	35.4	23.2	21.2	22.6	2.9	1.9 to 4.5	46.4	45.3	10.7	21.8	1.8	0.9 to 3.8
Total												
Population at risk, n	3,951	17,148	42,927	64,026			624	6,866	14,755	22,245		
No. of patients	25	44	82	151			6	22	19	47		
Person-years	58,018	250,025	639,171	947,215			7,439	82,715	180,465	270,619		
Crude rate†	43.1	17.6	12.8	15.9			80.7	26.6	10.5	17.4		
Crude RR	3.2	1.4	Ref				8.1	1.4	Ref			
95% CI*	2.0 to 5.0	1.0 to 2.0					3.1 to 18.0	0.7 to 2.6				

Abbreviations: RR, relative risk; Ref, reference.
*Analyses were performed using the Cox regression.
†The crude incidence was calculated as the total number of patients divided by person-years accumulated in each row and is presented per 100,000 person-years.

model,[3] we may speculate that hematopoietic stem cells of people exposed to higher radiation doses had more genetic damage than those of people exposed to lower dose or than those of the elderly population in general. However, we feel that the multistep pathogenesis model does not fully explain the recent increased risk of MDS. Chromosomal and genetic instabilities as consequences of targeted and/or nontargeted effects of radiation exposure[30] may play a role in the late development of MDS as well as solid cancers in atomic bomb survivors. In fact, we observed higher frequencies of complex karyotypic abnormalities, including random aneuploidies, among proximally exposed MDS patients in this study (Appendix Table A1). Another possible paradigm is the cancer stem-cell theory, including leukemic stem cells.[31,32] Trosko[33] suggests the role of organ-specific adult stem cells as the target cells for radiation-induced carcinogenesis, and the age-related changes in quality of the injured stem cells could affect cancer risks later in life. This concept may explain the long latency of MDS risk in atomic bomb survivors, although little is known about MDS stem cells.

This study has several limitations. Follow-up is limited and there is no information on MDS risks until 40 years after exposure. It was not possible to determine whether or not the incidence rate of MDS were elevated in the decades immediately after the bombings, since MDS was not recognized as a distinct entity until the mid-1980s. The dose-response analyses were performed for a small number of patients. The distance analyses did not account for variations in shielding among survivors, which would modify their actual doses. Information on dates of prior cancers and other prior chemotherapy or radiotherapy was not available for the ABDI data set.

As of 2007, we confirmed that 42 patients among the 151 ABDI-MDS patients progressed to overt leukemia (data not shown). Further studies are needed to clarify the effect of radiation on leukemic transformation as well as the nature of the radiation-induced MDS and the dose-response pattern. Efforts to expand the study to include MDS occurring among Hiroshima survivors are underway.

In conclusion, this study showed that acute radiation exposure is associated with increased risk of developing MDS later in life. This

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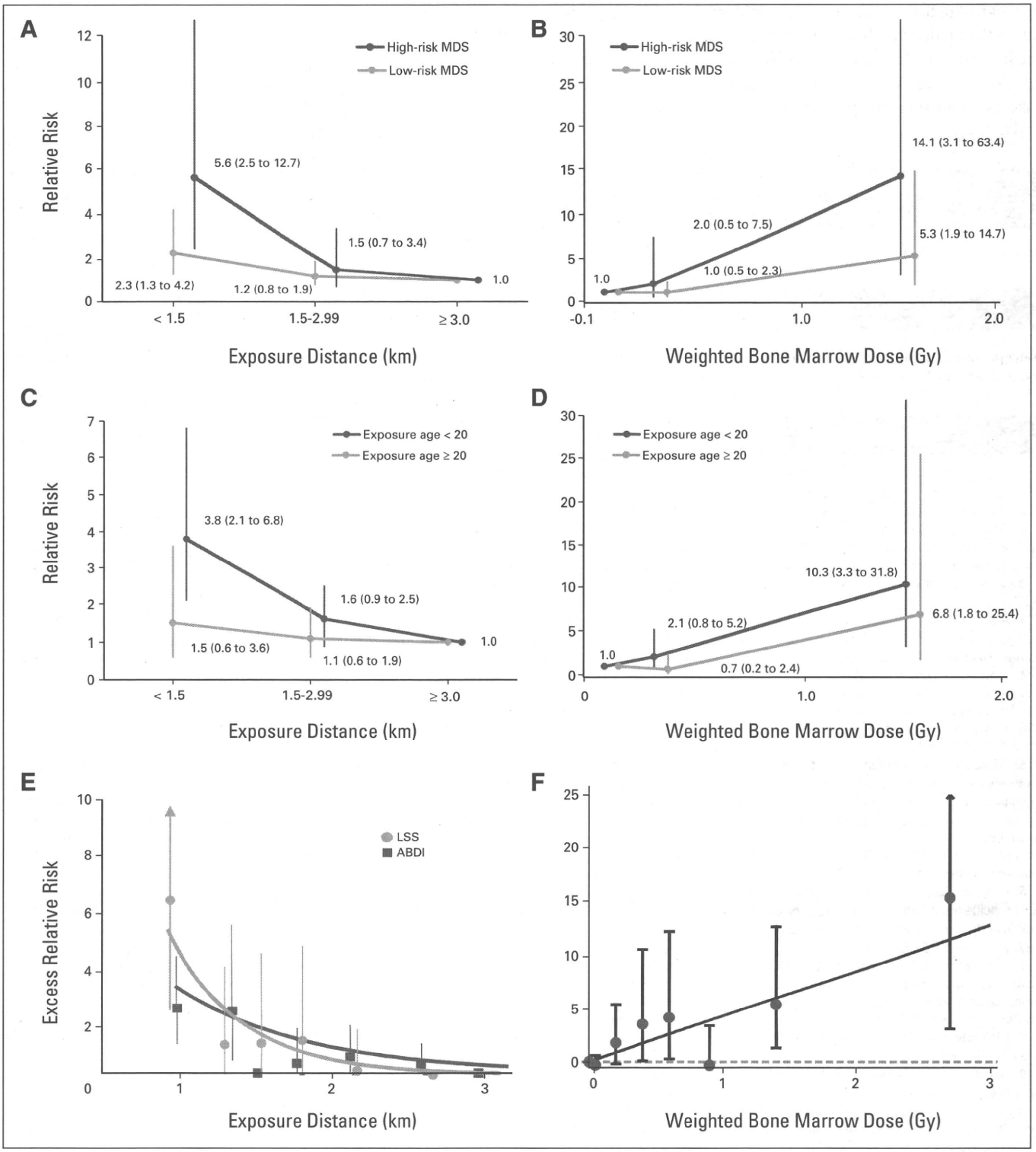

Fig 2. Risk of myelodysplastic syndromes (MDS) by exposure distance and dose. (A) Relative risks of MDS by French-American-British classification subtype in Atomic Bomb Disease Institute cohort, and (B) in Life Span Study-Nagasaki cohort. The high-risk MDS indicates French-American-British classification subtypes of refractive anemia with excess blasts and refractive anemia with excess blasts in transformation, and the low-risk MDS indicates the subtypes of refractive anemia and refractive anemia with ringed sideroblasts. (C) Relative risks of MDS by age at exposure in Atomic Bomb Disease Institute cohort, and (D) in Life Span Study-Nagasaki cohort. (E) Sex- and age-adjusted distance-response for MDS. The lines display the best-fitted excess relative risk curves based on distance category–specific relative risk. (F) Sex- and age-adjusted radiation dose-response for MDS. The line displays the best-fitted linear excess relative risk dose-response without risk modification based on dose category–specific relative risk. The dashed horizontal line represents excess relative risk = 0. Whiskers show the 95% CIs.

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suggests that radiation-induced MDS might involve a different pathogenesis than radiation-induced leukemia. Clinicians should perform careful long-term follow-up of people who have been exposed to radiation to detect MDS as early as possible and reduce the risk of leukemic transformation by using new drugs such as DNA hypomethylating agents.[34]

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

Conception and design: Masako Iwanaga, Dale L. Preston, Kazunori Kodama, Masao Tomonaga

Financial support: Masako Iwanaga, Masao Tomonaga

Administrative support: Akihiko Suyama, Kazunori Kodama, Masao Tomonaga

Collection and assembly of data: Masako Iwanaga, Midori Soda, Yumi Takasaki, Masayuki Tawara, Tatsuro Joh, Tatsuhiko Amenomori, Masaomi Yamamura, Yoshiharu Yoshida, Takashi Koba, Yasushi Miyazaki, Tatsuki Matsuo, Masao Tomonaga

Data analysis and interpretation: Masako Iwanaga, Wan-Ling Hsu, Midori Soda, Dale L. Preston, Akihiko Suyama, Masao Tomonaga

Manuscript writing: Masako Iwanaga, Wan-Ling Hsu, Midori Soda, Yumi Takasaki, Masayuki Tawara, Tatsuro Joh, Tatsuhiko Amenomori, Masaomi Yamamura, Yoshiharu Yoshida, Takashi Koba, Yasushi Miyazaki, Tatsuki Matsuo, Dale L. Preston, Akihiko Suyama, Kazunori Kodama, Masao Tomonaga

Final approval of manuscript: Masako Iwanaga, Wan-Ling Hsu, Midori Soda, Yumi Takasaki, Masayuki Tawara, Tatsuro Joh, Tatsuhiko Amenomori, Masaomi Yamamura, Yoshiharu Yoshida, Takashi Koba, Yasushi Miyazaki, Tatsuki Matsuo, Dale L. Preston, Akihiko Suyama, Kazunori Kodama, Masao Tomonaga

REFERENCES

1. Bennett JM, Catovsky D, Daniel MT, et al: Proposals for the classification of the myelodysplastic syndromes. Br J Haematol 51:189-199, 1982
2. Bowen DT: Etiology and epidemiology of MDS, in Deeg HJ, Bowen DT, Gore SD, et al (eds): Myelodysplastic Syndromes (Hematologic Malignancies). Berlin, Germany, Springer-Verlag, 2006, pp 15-22
3. Aul C, Bowen DT, Yoshida Y: Pathogenesis, etiology and epidemiology of myelodysplastic syndromes. Haematologica 83:71-86, 1998
4. Matsuo T, Tomonaga M, Bennett JM, et al: Reclassification of leukemia among A-bomb survivors in Nagasaki using French-American-British (FAB) classification for acute leukemia. Jpn J Clin Oncol 18:91-96, 1988
5. Tomonaga M, Matsuo T, Carter RL, et al: Differential effects of atomic bomb irradiation in inducing major leukemia types: Analyses of open-city cases including the Life Span Study cohort based upon update diagnostic systems and the dosimetry system 1986 (DS86). Radiation Effects Research Foundation Technical Report 9-91, 1993
6. Preston DL, Kusumi S, Tomonaga M, et al: Cancer incidence in atomic bomb survivors: Part III. Leukemia, lymphoma and multiple myeloma, 1950-1987. Radiat Res 137:S68-S97, 1994 (suppl 2)
7. Andersson M, Carstensen B, Visfeldt J: Leukemia and other related hematological disorders among Danish patients exposed to Thorotrast. Radiat Res 134:224-233, 1993
8. Moloney WC: Radiogenic leukemia revisited. Blood 70:905-908, 1987
9. Romanenko A, Bebeshko V, Hatch M, et al: The Ukrainian-American study of leukemia and related disorders among Chornobyl cleanup workers from Ukraine: I. Study methods. Radiat Res 170: 691-697, 2008
10. Gundestrup M, Klarskov Andersen M, Sveinbjornsdottir E, et al: Cytogenetics of myelodysplasia and acute myeloid leukaemia in aircrew and people treated with radiotherapy. Lancet 356:2158, 2000
11. Oda K, Kimura A, Matsuo T, et al: Increased relative risk of myelodysplastic syndrome in atomic bomb survivors. J Nagasaki Med Assoc 73:S174-S179, 1988
12. Albitar M, Manshouri T, Shen Y, et al: Myelodysplastic syndrome is not merely "preleukemia." Blood 100:791-798, 2002
13. Steensma DP: The spectrum of molecular aberrations in myelodysplastic syndromes: In the shadow of acute myeloid leukemia. Haematologica 92:723-727, 2007
14. Finch SC: Myelodysplasia and radiation. Radiat Res 161:603-606, 2004
15. Soda M, Ikeda T, Matsuo T, et al: Cancer incidence in Nagasaki Prefecture 1993-1997, in Parkin DM, Whelan SL, Ferlay J, et al (eds): Cancer Incidence in Five Continents, Vol. VIII. Lyon, France, International Agency for Research on Cancer/International Association of Cancer Registry, 2003, pp 390-393
16. Fritz A, Percy C, Jack A, et al: World Health Organisation: International Classification of Diseases for Oncology, 3rd Edition (ICD-O-3). Geneva, Switzerland, WHO, 2000
17. Iwanaga M, Tagawa M, Tsukasaki K, et al: Relationship between monoclonal gammopathy of undetermined significance and radiation exposure in Nagasaki atomic bomb survivors. Blood 113:1639-1650, 2009
18. Young RW, Kerr GD (eds): Reassessment of the Atomic Bomb Radiation Dosimetry for Hiroshima and Nagasaki, Dosimetry System 2002: Report of the Joint US-Japan Working Group. Hiroshima, Japan, Radiation Effects Research Foundation, 2005
19. Preston DL, Ron E, Tokuoka S, et al: Solid cancer incidence in atomic bomb survivors: 1958-1998. Radiat Res 168:1-64, 2007
20. Greenberg PL, Young NS, Gattermann N: Myelodysplastic syndromes. Hematology Am Soc Hematol Educ Program 136-161, 2002
21. Cullings HM, Fujita S, Funamoto S, et al: Dose estimation for atomic bomb survivor studies: Its evolution and present status. Radiat Res 166:219-254, 2006
22. Preston DL, Lubin JA, Pierce DA, et al: EPICURE User's Guide. Hirosoft International Corporation, Seattle, WA, 1993
23. Shimizu H, Matsushita Y, Aoki K, et al: Prevalence of the myelodysplastic syndromes in Japan. Int J Hematol 61:17-22, 1995
24. Harada H, Harada Y, Tanaka H, et al: Implications of somatic mutations in the AML1 gene in radiation-associated and therapy-related myelodysplastic syndrome/acute myeloid leukemia. Blood 101:673-680, 2003
25. Zharlyganova D, Harada H, Harada Y, et al: High frequency of AML1/RUNX1 point mutations in radiation-associated myelodysplastic syndrome around Semipalatinsk nuclear test site. J Radiat Res (Tokyo) 49:549-555, 2008
26. Pedersen-Bjergaard J, Andersen MT, Andersen MK: Genetic pathways in the pathogenesis of therapy-related myelodysplasia and acute myeloid leukemia. Hematology Am Soc Hematol Educ Program 392-397, 2007
27. Bernasconi P: Molecular pathways in myelodysplastic syndromes and acute myeloid leukemia: Relationships and distinctions—A review. Br J Haematol 142:695-708, 2008
28. Corey SJ, Minden MD, Barber DL, et al: Myelodysplastic syndromes: The complexity of stem-cell diseases. Nat Rev Cancer 7:118-129, 2007
29. Nimer SD: MDS: A stem cell disorder—But what exactly is wrong with the primitive hematopoietic cells in this disease? Hematology Am Soc Hematol Educ Program 43-51, 2008
30. Morgan WF: Non-targeted and delayed effects of exposure to ionizing radiation: II. Radiation-induced genomic instability and bystander effects in vivo, clastogenic factors and transgenerational effects. Radiat Res 159:581-596, 2003
31. Reya T, Morrison SJ, Clarke MF, et al: Stem cells, cancer, and cancer stem cells. Nature 414: 105-111, 2001
32. Hope KJ, Jin L, Dick JE: Acute myeloid leukemia originates from a hierarchy of leukemic stem cell classes that differ in self-renewal capacity. Nat Immunol 5:738-743, 2004
33. Trosko JE: Concepts needed to understand potential health effects of chronic low-level radiation exposures: Role of adult stem cells and modulated cell-cell communication. International Congress Series 1299:101-113, 2007
34. Silverman LR, McKenzie DR, Peterson BL, et al: Further analysis of trials with azacitidine in patients with myelodysplastic syndrome: Studies 8421, 8921, and 9221 by the Cancer and Leukemia Group B. J Clin Oncol 24:3895-3903, 2006

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ORIGINAL ARTICLE

Safety and efficacy of the terminal complement inhibitor eculizumab in Japanese patients with paroxysmal nocturnal hemoglobinuria: the AEGIS Clinical Trial

Yuzuru Kanakura · Kazuma Ohyashiki · Tsutomu Shichishima · Shinichiro Okamoto · Kiyoshi Ando · Haruhiko Ninomiya · Tatsuya Kawaguchi · Shinji Nakao · Hideki Nakakuma · Jun-ichi Nishimura · Taroh Kinoshita · Camille L. Bedrosian · Marye Ellen Valentine · Gus Khursigara · Keiya Ozawa · Mitsuhiro Omine

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Abstract Paroxysmal nocturnal hemoglobinuria (PNH) is a progressive and life-threatening disease characterized by complement-mediated chronic hemolysis, resulting in serious life-threatening complications and early mortality. Eculizumab, a humanized anti-C5 monoclonal antibody that inhibits terminal complement activation, has been shown to reduce hemolysis in PNH patients. The pivotal open-label, 12-week phase II registration study (AEGIS) was designed to evaluate the efficacy and safety of eculizumab in Japanese patients with PNH. This trial achieved its primary endpoint of reducing intravascular hemolysis with high statistical significance. Twenty-seven of the 29 patients responded to eculizumab treatment, resulting in an 87% reduction in hemolysis ($P < 0.0001$) and subsequent improvement in anemia ($P = 0.0003$) despite reduction in transfusion requirements ($P = 0.006$). Fatigue and dyspnea significantly improved within 1–2 weeks of eculizumab treatment and the improvement was independent of changes in hemoglobin. Chronic kidney disease (CKD) was common (66%) and eculizumab treatment improved CKD in 41% of patients at 12 weeks ($P < 0.001$). Elevated thrombotic risk was evident in Japanese PNH patients and eculizumab treatment normalized D-dimer levels in 45% of patients with elevated D-dimers at baseline ($P < 0.001$). The AEGIS results demonstrate that eculizumab is effective, safe and well tolerated in Japanese patients with PNH.

Y. Kanakura (✉) · J. Nishimura
Department of Hematology and Oncology,
Osaka University Hospital, Suita, Japan
e-mail: kanakura@bldon.med.osaka-u.ac.jp

K. Ohyashiki
Tokyo Medical University Hospital, Tokyo, Japan

T. Shichishima
Fukushima Medical University, Fukushima, Japan

S. Okamoto
Division of Hematology,
Keio University School of Medicine, Tokyo, Japan

K. Ando
Department of Hematology and Oncology,
Tokai University, Isehara, Japan

H. Ninomiya
University of Tsukuba, Tsukuba, Japan

T. Kawaguchi
Kumamoto University, Kumamoto, Japan

S. Nakao
Cellular Transplantation Biology,
Kanazawa University, Kanazawa, Japan

H. Nakakuma
Department of Hematology/Oncology,
Wakayama Medical University, Wakayama, Japan

T. Kinoshita
Research Institute for Microbial Diseases,
Osaka University Hospital, Suita, Japan

C. L. Bedrosian · M. E. Valentine · G. Khursigara
Alexion Pharmaceuticals, Cheshire, CT, USA

G. Khursigara
e-mail: khursigarag@alxn.com

K. Ozawa
Jichi Medical University Hospital, Tochigi, Japan

M. Omine
Showa University Fujigaoka Hospital, Yokohama, Japan

Keywords Paroxysmal nocturnal hemoglobinuria · Complement-inactivating agents · Hemolysis · Eculizumab · Hematopoietic stem cell

1 Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is a progressive and life-threatening disease characterized by chronic hemolysis [20, 28, 36]. The 5-year mortality in patients presenting with hemolysis ranges from 15 to 35% and median survival ranges from 10 to 22 years after diagnosis [10, 20, 27, 36]. The disease can present at any age with the median age ranging from early 30s to mid-40s [27]. PNH arises from an acquired genetic mutation in the X-linked phosphatidylinositol glycan-complementation class A (*PIGA*) of hematopoietic progenitor cells leading to clonal deficiency of glycosylphosphatidylinositol (GPI)-linked proteins on the surface membrane of blood cells [38, 41]. This GPI deficiency results in the loss of the complement inhibitor proteins CD55 and CD59 from the surface of hematopoietic cells in PNH patients leading to complement-mediated red cell lysis [3, 28], platelet activation [2], and hemostatic activation with inflammation [40].

Historically, physicians viewed and treated PNH as a disease of anemia. However, the demonstration of the underlying phenotype—deficiency of GPI-linked complement inhibitors CD55 and CD59—indicates that the primary clinical manifestation is the terminal complement activation causing not only lysis of PNH red blood cells but also in parallel platelet, monocyte, and leukocyte activation with consequent inflammation and hemostatic activation [13, 40]. Thus, anemia is only a single consequence of the underlying chronic intravascular hemolysis. Patients suffer severe morbidities and early mortality as a direct result of terminal complement activation with chronic hemolysis including kidney disease, thrombosis, pulmonary hypertension, hemoglobinuria, debilitating fatigue, severe dyspnea, disabling pain, and a poor quality of life (QoL) [15, 18, 27–29, 31, 32]. Thromboembolism (TE) accounts for 40–67% of PNH-related deaths and renal failure accounts for 8–18% of PNH-related deaths [15, 18, 27–29, 31, 32].

Eculizumab is a humanized monoclonal antibody that specifically targets the terminal complement protein C5, thereby inhibiting terminal complement-mediated hemolysis [33]. The efficacy and safety of eculizumab have been evaluated in two multinational phase III studies and a multinational extension trial performed predominantly in the North America, Europe and Australia [4, 21, 22]. These studies demonstrate that eculizumab significantly reduces hemolysis, thrombotic events, renal impairment, pulmonary hypertension, and improves fatigue, QoL, and anemia, while reducing transfusion requirements.

While the clinical course of PNH in both the Western and Asian populations is associated with similar mortality rates and both populations suffer significant hemolysis-mediated symptoms, the clinical manifestation of the disease is perceived to differ between the two populations [27]. The current study was an open-label, single-arm, phase II registration study (AEGIS) designed to evaluate the safety, efficacy, pharmacokinetics and pharmacodynamics of eculizumab in Japanese patients with PNH and to examine the consistency of these results with the previously reported multinational phase III and extension studies of eculizumab.

2 Methods

AEGIS was an open-label, single-arm, multi-center study in Japanese patients who were 12 years of age or older with a diagnosis of PNH for at least 6 months and was conducted at 9 medical centers in Japan. Additional inclusion criteria included: the presence of a population of GPI-deficient red blood cells (PNH Type III RBCs) by flow cytometry $\geq$ 10% at screening; and lactate dehydrogenase (LDH) $\geq$ 1.5 times the upper limit of normal (ULN) within 12 weeks of screening or during the screening period, and platelet count $\geq 30 \times 10^9$/L. Enrolled patients were judged by the physician to require at least one red blood cell transfusion within the past 2 years, although enrollment of patients who had received no red blood cell transfusions was permitted provided that the physician had judged the patient to have required transfusion. All patients were required to give written informed consent. Females of child-bearing potential were required to have a negative pregnancy test (serum HCG) at screening. Sexually active females had to agree to use a reliable and medically approved method of contraception. Exclusion criteria included: platelet count $< 30 \times 10^9$/L at screening; absolute neutrophil count $\leq$ 500/μL at screening; known or suspected hereditary complement deficiency; history of hematopoietic stem cell transplantation; and participation in any other investigational drug trial or exposure to other investigational agent, device, or procedure within 30 days prior to screening. Patients who were pregnant or breast-feeding, or who could become pregnant or intended to conceive during the course of the study (including the post-treatment period and the follow-up visits for early termination) were excluded. Additional exclusion criteria included a history of meningococcal disease and, in the opinion of the investigator, the presence or suspicion of active bacterial infection 2 weeks prior to first dose of eculizumab, or recurrent bacterial infections.

Patients received 600 mg of eculizumab intravenously every 7 ± 2 days for 4 weeks, followed by 900 mg 1 week

later followed by 900 mg every 14 ± 2 days for a total of 12 weeks. All patients were vaccinated with a meningococcal vaccine at least 2 weeks before receiving the first dose of eculizumab.

The primary efficacy endpoint in the AEGIS study was the change in intravascular hemolysis (as measured by change in LDH) at study Week 12 from baseline. Secondary endpoints included change from baseline in the Functional Assessment of Chronic Illness Therapy-Fatigue (FACIT-Fatigue) [5] scale at study Week 12, change in PNH Type III RBC count at study Week 12, change in transfusion requirements (number of units of packed RBCs transfused), change in plasma-free hemoglobin (free-Hgb) at study Week 12, area under the curve (AUC) for change of LDH, and change in the European Organization for Research and Treatment of Cancer Quality of Life Core 30 (EORTC QLQ-C30) [1] questionnaire score at study Week 12. D-Dimer levels were measured at the central laboratory at baseline, Week 4 and Week 12.

The effect of eculizumab on renal function as measured by an improvement or worsening in chronic kidney disease (CKD) stage during treatment was also evaluated. CKD stages were determined for each patient according to Kidney Disease Outcomes Quality Initiative (KDOQI) CKD published guidelines classification (Stage 5: glomerular filtration rate, GFR < 15 mL/min/1.73 m^2; Stage 4: GFR 15–30 mL/min/1.73 m^2; Stage 3: GFR 30–60 mL/min/1.73 m^2; Stage 2: GFR 60–90 mL/min/1.73 m^2 and evidence of proteinuria; Stage 1: GFR ≥ 90 mL/min/1.73 m^2 and evidence of proteinuria; no CKD: GFR > 60 mL/min/1.73 m^2 and no evidence of proteinuria) [26]. An improvement in renal function was defined as a categorical reduction in CKD stage level or fulfilling the criteria of no CKD. Worsening in renal function was defined as a categorical increase in CKD stage level.

Evaluation of the safety of eculizumab included assessment of adverse events (AEs), assessment of thrombotic events, laboratory measurements, vital signs, ECG, and chest X-ray.

The pharmacokinetics of eculizumab was determined with a validated enzyme-linked immunosorbent assay that detects both free and C5-bound eculizumab [39]. The analytical range of the assay was 10–640 μg/mL. The pharmacodynamics of eculizumab was determined by measuring the capacity of the patient's serum to lyse chicken erythrocytes in a validated standard total human serum-complement hemolytic assay [30].

2.1 Statistical analysis

Changes in LDH, hemoglobin (Hgb), RBC type III, free-Hgb and PRBC transfusion units from baseline to Week 12 were analyzed by Wilcoxon's signed rank test. Transfusion avoidance was evaluated with a McNemar test.

Changes in scores on the FACIT-Fatigue instrument and the EORTC QLQ-C30 instrument from baseline through Week 12 were analyzed with the use of a mixed-effects model, with baseline scores as the covariate, time as the fixed effect, and the patient identifier as the random effect. The proportion of patients returning to normal range of D-dimer was analyzed by exact binomial test.

Changes in the proportion of patients in each CKD stage from baseline were compared using Chi-square analyses and the hypothesis tested the probability of worsening CKD stage was equal to the probability of improving CKD stage.

For each subgroup of patients with a history of bone marrow dysfunction (BMD) that includes aplastic anemia (AA) or myelodysplastic syndrome (MDS), or no history of BMD, the change in LDH, Hgb, FACIT-Fatigue, EORTC from baseline was analyzed by a t test in mixed-effects model and between the two subgroups was analyzed by F test in mixed-effects model with baseline as covariate, subgroup and time as fixed effect, and patient as random effect.

3 Results

3.1 AEGIS patient characteristics

Eculizumab was administered to 29 Japanese patients (14 men and 15 women; median patient age 47 years; range 26–70 years) at 9 institutions (see Table 1). Forty-five percent (45%; 13/29) of patients had a history of AA or MDS. Forty-eight percent (48%; 14/29) of patients were receiving concomitant corticosteroids. The cohort also included 2 patients who were never transfused. These two patients demonstrated clinical signs and symptoms of PNH comparable to patients who had received transfusions. Twenty-seven of 29 patients completed the study.

3.2 Hemolysis

The primary endpoint—reduction of intravascular hemolysis—was achieved with a high level of statistical significance with eculizumab treatment. Eculizumab treatment reduced LDH 87% from a median of 1814 U/L at baseline to 244 U/L at 12 weeks of treatment ($P < 0.0001$; normal range 103–223 U/L). Mean LDH levels decreased from 1845 ± 115 U/L at baseline to 399 ± 99 U/L at 12 weeks (Table 1). A significant reduction in LDH was observed within 1 week of treatment ($P < 0.0001$) and this reduction was sustained throughout the 12-week study (Fig. 1). In two patients, eculizumab serum concentrations were maintained above the effective level of 35 μg/ml but

Table 1 Baseline characteristics, effects of eculizumab on hematologic parameters

Parameter	Baseline
Median age (years) (range)	47 (26–70)
Gender, female	52%
History of AA or MDS	45%
History of thrombosis	17%
Elevated D-dimer	38%
Elevated D-dimer or thrombosis	52%
Chronic kidney disease (CKD)	66%
Concomitant antithrombotic (%)	31%
Concomitant steroids (%)	48%

Parameter	Mean ± SE (median)		*P* value
	Baseline	12 weeks	
LDH (U/L)	1845 ± 115 (1814)	399 ± 100 (244)	<0.0001
RBC type III (%)	43.9 ± 4.5 (39.2)	57.3 ± 4.9 (56.7)	<0.0001
PNH RBC mass ($\times 10^{12}$/μL)	1.2 ± 0.1 (1.2)	1.8 ± 0.2 (1.7)	<0.0001
Transfusion (units/12 weeks)	5.2 ± 1.0 (2.0)	1.5 ± 0.7 (0.0)	0.006
Hemoglobin (g/dL)	7.9 ± 0.3 (7.6)	8.9 ± 0.4 (9.0)	0.0003
Free hemoglobin (mg/dL)	22.6 ± 2.6 (20.0)	2.8 ± 1.0 (1.0)	<0.0001

P value was calculated using signed rank test

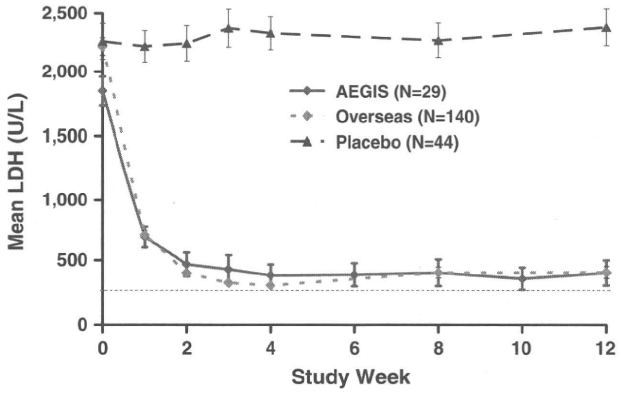

Fig. 1 Comparison of the effects of eculizumab on LDH in AEGIS versus multinational studies (units, U/L). Treatment with eculizumab reduced mean LDH levels from baseline (Week 0) within 1 week of treatment ($P < 0.001$ from baseline) and was sustained for 12 weeks ($P < 0.001$ from baseline) in Japanese-treated patients (AEGIS, *red diamonds*). The 87% reduction in the Japanese patients was similar to the treated patients in multinational overseas study ($P < 0.001$; overseas, *orange diamonds*). Patients not treated with eculizumab did not demonstrate a reduction in LDH (placebo, *blue triangles*). The overseas data consist of the placebo-controlled TRIUMPH and open-label SHEPHERD registration studies [4, 21, 22]. The placebo group is from the TRIUMPH study

without reduction in LDH, although a partial drug response was observed with a reduction in complement activation as measured by in vitro CH50 assays.

3.3 Red blood cell mass and anemia

Eculizumab treatment significantly increased the proportion of PNH type III RBCs from a median of 39.2% at baseline to 56.7% at Week 12 ($P < 0.001$; Table 1). Similarly, the proportion of type II RBCs was also significantly increased from a median of 4.2% at baseline to a median of 5.6% at Week 12 ($P < 0.0001$). Eculizumab treatment significantly increased PNH RBC mass from a mean at baseline 1.2×10^{12}/μL to 1.8×10^{12}/μL at 12 weeks ($P < 0.0001$; Table 1).

Eculizumab treatment significantly reduced the number of PRBC transfusion units from a median 2.0 units (mean of 5.2 ± 1.0 units) per patient in the 12 weeks prior to eculizumab treatment to a median 0.0 units (mean 1.5 ± 0.7) per patient during the 12-week treatment phase ($P = 0.006$; Table 1). Of the 21 patients that had received at least one or more transfusion in the 12-week period prior to treatment, 66% (14/21) did not require transfusion during the 12 weeks of treatment ($P = 0.001$). Despite the decrease in transfusion requirements, Hgb levels increased from a median of 7.6 g/dL at baseline to 9.0 g/dL at Week 12 ($P = 0.0003$; Table 1). However, the statistical improvement in Hgb was first observed 8 weeks after treatment initiation (median 8.7 g/dL at Week 8 vs. 7.6 g/dL at baseline, $P = 0.007$).

Table 2 Effects of eculizumab on fatigue, dyspnea, hemoglobin and free hemoglobin during first 4 weeks of treatment

	Baseline	Change from baseline [mean ± SE, median (*P* value)]			
		Week 1	Week 2	Week 4	Week 12
Fatigue (FACIT Score)	38.5 ± 1.9, 41.0	2.1 ± 1.1, 2.0 (0.04)	4.2 ± 1.0, 4.0 (<0.001)	4.9 ± 1.4, 4.0 (<0.001)	4.1 ± 2.3, 5.0 (<0.001)
Dyspnea (EORTC Score)[a]	37.9 ± 5.2, 33.3	−11.5 ± 4.1, 0.0 (0.006)	−13.8 ± 3.9, 0.0 (<0.001)	−13.8 ± 4.5, 0.0 (<0.001)	−13.8 ± 4.5, 0.0 (<0.001)
Hemoglobin (g/dL)	7.9 ± 0.3, 7.6	N/A	0.2 ± 0.2, 0.1 (NS)	0.4 ± 0.2, 0.25 (NS)	1.0 ± 0.25, 1.0 (<0.001)
Free hemoglobin (mg/dL)	22.6 ± 2.6, 22	−18.3 ± 2.7, −12.0 (<0.001)	−18.6 ± 3.0, −17.0 (<0.001)	−11.9 ± 9.1, −16.0 (0.006)	−19.8 ± 2.7, −17.0 (<0.001)

NS not significant: *P* value based on *t* test

[a] A negative change in EORTC score of fatigue and dyspnea indicates improvement

There was an immediate reduction in median free-Hgb levels at 1 week of eculizumab treatment (from 20.0 mg/dL at baseline to 4.2 mg/dL; $P < 0.001$) compared to baseline levels and this reduction was sustained throughout the study to 1.0 mg/dL at Week 12 ($P < 0.0001$).

3.4 Improvement in FACIT-Fatigue and QoL

Fatigue levels, as measured by the FACIT-Fatigue instrument and confirmed by the EORTC-Fatigue instrument, significantly improved within 2 weeks of eculizumab treatment. Thirty-eight percent of treated patients experienced a clinically meaningful improvement in fatigue (at least a 3-point increase on the FACIT-Fatigue scale) [6, 7] at Week 1 of treatment, 62% at Week 2 and 66% at Week 12. Eculizumab treatment improved fatigue, a mean of 2.1 and 4.2 points on the FACIT-Fatigue scale after 1 and 2 weeks of treatment ($P = 0.04$ and $P < 0.001$ compared to baseline, respectively; Table 2). The improvement in fatigue was sustained, a mean increase of 4.1 points at 12 weeks ($P < 0.001$). The rapid improvement in fatigue measured by FACIT-Fatigue was confirmed by a large mean improvement in EORTC QLQ-C30 Fatigue of 7.1 and 11.1 points by Weeks 1 and 2 ($P < 0.001$ compared to baseline each week).

Treatment with eculizumab demonstrated improvements in QoL ($P = 0.02$) as measured by EORTC QLQ-C30, with statistically significant improvements in global health status ($P = 0.02$), role ($P < 0.001$), physical ($P = 0.02$) and emotional functioning ($P = 0.002$), fatigue ($P < 0.0001$), dyspnea ($P < 0.0001$), and appetite loss symptoms ($P < 0.0001$). Fifty percent of Japanese patients treated with eculizumab improved by at least 10% in global health status at Week 12, a degree of improvement considered clinically meaningful. Eculizumab treatment was associated with a large and rapid improvement in patient-reported dyspnea symptoms with an 11.5 points improvement at Week 1 ($P = 0.02$). Furthermore, 41% of patients reported a major improvement (10% or greater) in dyspnea with eculizumab treatment that was sustained through Week 12.

3.5 Improvement in CKD

Renal dysfunction was common in the study population, with 66% (19 of 29 patients; Table 1; Fig. 3a) of patients demonstrating CKD at baseline. Patients treated with eculizumab for 12 weeks were more likely to improve (41%; 12/29) rather than worsen (3%; 1/29) CKD stage ($P = 0.0002$ improved compared to worsen) and 55% (16/29) of patients had no change in their CKD stage (Fig. 3b). Of the 16 patients with CKD Stage 1–2 at baseline, 11 patients (69%) improved with eculizumab; of the 3 patients with CKD Stage 3–5 at baseline, 1 patient (33%) improved with eculizumab treatment (Fig. 3).

3.6 Thrombotic events and D-dimer levels

There were five patients with a history of TEs (one patient had a cerebrovascular accident and 4 patients had a deep vein thrombosis) prior to study enrollment and there were no reported TEs during eculizumab treatment. At baseline, 11/29 (38%) of patients had D-dimer levels above the ULN. Seven of the 11 patients with elevated D-dimer levels had evidence of CKD. Eculizumab treatment was associated with the normalization of D-dimer levels in 5 of the 11 (45%) of patients with elevated D-dimer ($P < 0.001$).

3.7 AA or MDS subpopulation analysis

Forty-five percent (13/29) of the PNH patients enrolled in the study were also diagnosed with a history of BMD (AA or MDS). Patients with or without BMD showed similar levels of hemolysis, as measured by LDH ($P = 0.74$ between both subpopulations), Hgb ($P = 0.60$), transfusions ($P = 0.82$), FACIT-Fatigue score ($P = 0.66$),

EORTC-Fatigue ($P = 0.62$), and EORTC-Dyspnea ($P = 0.32$) at study entry. Both PNH patient subgroups showed an immediate and sustained reduction in LDH from baseline within the first week of treatment ($P < 0.001$ in each group). Eculizumab treatment improved QoL measures in both patient groups as indicated by significant improvements in FACIT-Fatigue ($P < 0.001$ and $P = 0.008$, respectively), EORTC-Fatigue ($P = 0.001$ and $P = 0.008$, respectively) and EORTC-Dyspnea ($P < 0.001$ and $P = 0.003$, respectively). Hgb was improved with eculizumab treatment at Week 12 from baseline in both groups ($P < 0.05$ and $P = 0.006$, respectively). There was no difference in improvement between the two groups during eculizumab treatment: LDH reduction ($P = 0.51$), increase Hgb ($P = 0.26$), improvement in FACIT-Fatigue ($P = 0.95$), EORTC-Global health ($P = 0.90$), EORTC-Fatigue ($P = 0.94$), and EORTC-Dyspnea ($P = 0.49$).

PNH patients with PNH and BMD experienced significant improvement in renal function. At baseline, 12/13 (92%) of patients with a history of BMD demonstrated CKD. Eculizumab treatment led to 7/13 patients improving CKD, 6/13 patients with no change, and no patients with worsening of CKD ($P = 0.0001$). At baseline, 7/16 (44%) of patients without a history of BMD demonstrated CKD. Eculizumab treatment was associated with a strong trend for improvement ($P = 0.07$) with 5/16 patients improving to a level of no CKD, 10/16 patients with no change, and one patient with worsening of CKD from Stage 0 to Stage 1.

3.8 Never transfused patients

Despite 2 patients never being transfused at baseline, these patients were hemolytic (LDH approximately 7- and 11-fold above normal, respectively), demonstrated significant organ damage with evidence of renal disease (CKD stage 2 and 1, respectively) and thrombosis (1 patient with DVT), and suffered disabling QoL as measured by FACIT-Fatigue and EORTC QLQ-C30 Dyspnea. In both patients, eculizumab treatment resulted in substantial 78–88% reductions in LDH, significant improvements in fatigue (improvements of 5 and 23 points, respectively), improvement in dyspnea in one patient (improvement of 33 points from baseline), and elimination of CKD with no subsequent TE in both patients.

3.9 Pharmacokinetics and pharmacodynamics of eculizumab

Blood samples for PK/PD assessments were collected at all dosing visits in the AEGIS study. Pharmacokinetic analysis showed that eculizumab trough levels reached a median of 85.8 μg/mL (range 20.4–172.5 μg/mL) and peak levels reached a median of 189.9 μg/mL (range 90.6–297.9 μg/mL) at study Week 2. Over the course of the study, both peak and trough levels were maintained above the levels reached at Week 2. No patients at Week 4, and 1 patient each at Weeks 6, 8, and 12 showed serum eculizumab levels below 35 μg/mL, a minimal level required to completely inhibit complement-related hemolysis in serum samples [17]. After the induction period (study Week 8), 93.1% (27/29) of patients showed strong inhibition of hemolysis.

3.10 Safety

Eculizumab was safe and well tolerated in all patients. The majority of AEs (98.3%) were reported as mild or moderate. There were no patient deaths during the study, and no patients withdrew participation due to an AE. There was a single infection-related serious AE (pyrexia) which was not reported as probably or definitely related to drug.

The most frequent treatment-emergent AEs were headache (52%), nasopharyngitis (41%), and nausea (21%). Notably, of 15 patients who reported headache, 14 did so within 1 day of study drug infusion. All headaches were reported as mild or moderate in severity and were effectively treated with over-the-counter medications. The frequency of headaches reduced from 45% during the first 4 weeks to only 14% during the following 8 weeks. Other AEs that were reported with ≥10% incidence were diarrhea (14%), eczema (10%), pyrexia (10%), and vomiting (10%) (Table 3). Most (88%) infection-related AEs were mild and no infection-related events were reported as probably or definitely related to drug. No meningococcal infections were reported during the treatment period. There were no deaths and no pregnancies, major adverse vascular events, or serious hemolysis events during the study.

Table 3 Frequently (≥10%) reported treatment-emergent adverse events

Preferred term	Patients, *n* (%)	
	Eculizumab ($N = 29$)	Placebo[a] ($N = 44$)
Total patient reporting	28 (96.6%)	37 (84.1%)
Serious AE	1 (3.5%)	5 (11.4%)
Withdrawal due to AE	0 (0.0%)	0 (0.0%)
AEs that were mild or moderate	(98.3%)	(97.0%)
Headache	15 (51.7%)	9 (20.5%)
Nasopharyngitis	12 (41.4%)	5 (11.4%)
Nausea	6 (20.7%)	3 (6.8%)
Diarrhea	4 (13.8%)	3 (6.8%)
Eczema	3 (10.3%)	0 (0%)
Pyrexia	3 (10.3%)	2 (4.5%)
Vomiting	3 (10.3%)	3 (6.8%)

[a] First 12 weeks of placebo from the multinational study

4 Discussion

The AEGIS trial results demonstrate that eculizumab is safe, effective and well tolerated in Japanese patients with PNH. The primary endpoint of the study, reduction of hemolysis, was achieved with a high level of statistical significance, demonstrating that treatment with eculizumab significantly suppresses chronic intravascular hemolysis 87% in Japanese patients with PNH. The response to eculizumab was immediate (within 1 week of treatment) and sustained for at least the current 12-week observation period (Fig. 1). These results are consistent with those of the multinational phase III trials of eculizumab which showed a similar 87% reduction in LDH from a median of 2042 U/L at baseline to 265 U/L at 12 weeks ($N = 140$; $P < 0.001$). The reduction in hemolysis in the multinational trials was sustained for at least the 54-month observation period in these studies (2165 U/L at baseline to 274 U/L at 18 months, $P < 0.001$, $n = 171$ and 277 U/L at 54 months, $P = 0.002$, $n = 10$) [37]. Given the globally consistent response to therapy in other multinational studies, it is expected that long-term eculizumab treatment will continue to result in sustained inhibition of hemolysis and reduction in the hemolysis-driven morbidities in Japanese patients.

At baseline, and despite ongoing immunosuppressive therapy in 4/13 patients, levels of hemolysis, fatigue, dyspnea, global health, CKD, anemia, and transfusion requirements were similar in patients with or without a history of AA or MDS. These results demonstrate that intravascular hemolysis significantly contributes to disabling symptoms in patients with PNH, irrespective of whether the patient has or does not have a history of AA or MDS. Further, our data demonstrate that chronic treatment with eculizumab effectively suppressed intravascular hemolysis and significantly improved signs and symptoms of PNH similarly in patients with or without a history of AA or MDS.

The immediate and sustained improvements in fatigue in Japanese PNH patients treated with eculizumab are similar to the improvements observed in the multinational trials. We observed that the significant improvements in fatigue are independent of any improvement in anemia, since the fatigue improvement at Week 1 preceded any observable change in Hgb levels (which did not change until Week 8, Fig. 2) [22]. The burden of fatigue in PNH is frequently underappreciated, and has historically been ascribed to concomitant anemia. The results of the current study as well as the parallel results of the multinational studies demonstrate, however, that in both Japanese and non-Japanese patients with PNH, terminal complement activation leading to intravascular hemolysis causes fatigue, and that fatigue in patients with PNH is independent of the level of

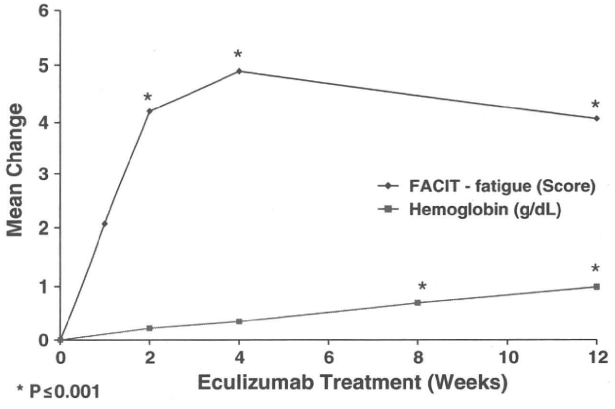

Fig. 2 Effects of eculizumab on FACIT-Fatigue scores. Treatment with eculizumab improved fatigue (as measured by FACIT-Fatigue) within 2 weeks of treatment ($P < 0.001$; *blue diamonds*). A change of 3 points is considered clinically significant. Significant improvement in hemoglobin was not seen in until Week 8 of treatment (*red squares*)

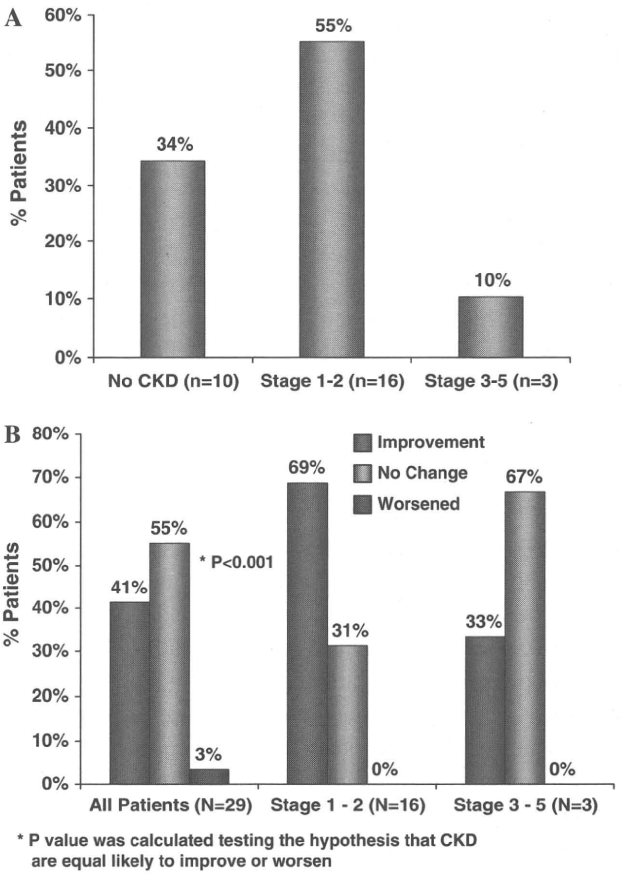

Fig. 3 Baseline and change in chronic kidney disease after first 12 weeks of eculizumab treatment. **a** CKD at baseline. **b** Treatment with eculizumab improved CKD in 41% of all patients ($P < 0.001$). 69% of patients with CKD Stage 1–2 at baseline improved and 33% of patients with CKD Stage 3–5 at baseline improved with eculizumab treatment

anemia. Hence, in patients with PNH, Hgb level alone will not accurately reflect the full burden of the disease.

Similar to fatigue, eculizumab treatment was associated with rapid improvements in dyspnea (within 1 week of eculizumab treatment, $P = 0.006$ from baseline) independent of anemia, as dyspnea improved well before any observable changes in Hgb in Japanese PNH patients (Table 2). Dyspnea is considered a manifestation of pulmonary hypertension and cardiac overload. N-terminal pro-brain natriuretic peptide (NT-proBNP), a measure of pulmonary vascular resistance and right ventricular dysfunction, is elevated in PNH patients, possibly due to elevated hemolysis and subsequent NO depletion [14, 15]. Treatment with eculizumab has been shown to reduce NT-proBNP and the reduction correlated with an increase of NO availability and improvement in dyspnea, both independent of anemia [14, 15]. Taken together, these data further demonstrate that symptoms historically associated with anemia such as fatigue and dyspnea are in fact independent of Hgb levels in patients with PNH. The reduction of terminal complement activation and chronic intravascular hemolysis appears to be directly responsible, independent of any possible improvement in anemia, for significantly reducing the severe morbidities in patients with PNH.

Transfusion requirement does not accurately reflect the disease burden or clinical risks associated with PNH. In our cohort, 2 Japanese patients had never been transfused yet demonstrated hemolysis comparable to patients that had been transfused including evidence of hemolysis, TE and CKD. The response to eculizumab in these never transfused patients was comparable to the benefits obtained with eculizumab treatment of patients who had been previously transfused in our study. Consistent with our data, a separate multinational study demonstrated that never-transfused PNH patients experience elevated hemolysis (median LDH of 1360 U/L) and that 87% had impaired QoL as documented by the patient or physician and 28% of the multinational group had clinical evidence of thrombosis [24]. Eculizumab treatment significantly reduced hemolysis and was associated with improved QoL, and reductions in thrombotic events, comparable to the results observed with treatment of the never-transfused Japanese patients in the current study. Taken together, it is apparent that hemolysis drives the signs and symptoms of PNH in both Japanese and multinational PNH patients independent of transfusions and transfusions do not appear to be a useful measure of the risks or clinical burden suffered by PNH patients.

Renal failure is a consequence of hemolysis and has a significant impact on survival in Japanese patients with PNH, accounting for 18% of deaths [27]. The incidence of renal failure is reported at 10.5% in the Japanese PNH population, similar to that reported in the US PNH population 9.6% [27]. Repetitive exposure to elevated cell-free Hgb causes renal hemosiderin accumulation, tubulo-interstitial inflammation, and kidney damage [25]. In addition, NO depletion due to excess free-Hgb leads to alterations in renal blood flow and can have a direct effect on the GFR and renal plasma flow [11, 12, 34, 35]. There is also evidence of microscopic infarction playing a role in chronic renal failure [9, 19]. We found that 37% (7/19) of patients with CKD had elevated D-dimer levels, suggestive that microthrombotic infarctions may also contribute to CKD, but only in some PNH patients. Elevated D-dimer levels are not specific to CKD patients as elevated D-dimer levels were evident in 40% (4/10) of patients with no CDK. We determined that renal dysfunction or damage, as defined by stages of CKD, is common (66%) in Japanese PNH patients enrolled in the study (Fig. 3), similar to the 64% of PNH patients with CKD in the PNH multinational studies and to the 68% of PNH patients with reduced creatinine clearance studied separately [9, 19]. Treatment with eculizumab led to improvement of CKD in 41% of Japanese patients at 12 weeks. While there is no placebo control arm in the current study, the placebo group in multinational PNH did not demonstrate any likelihood of CKD improvement compared to baseline ($P = 0.78$) at 26 weeks [19, 23]. Treatment of Japanese patients with milder CKD Stage 1–2 at baseline was associated with a higher likelihood of improvement in renal function. This result is similar to the PNH multinational trials in which 64% with Stage 1–2 at baseline improved with eculizumab treatment. There were very few patients in the current trial with CKD Stage 3–5 to determine the beneficial impact, although 1 of the 3 patients improved with eculizumab treatment, and no patient worsened. In the PNH multinational clinical trials, 20% of PNH patients with CKD Stage 3–5 showed improvement and 75% remained stable over 18 months. Taken together, these data suggest that eculizumab had a pronounced and beneficial effect on pre-existing CKD within 12 weeks of chronic treatment initiation and initiation of eculizumab treatment earlier in the disease course was more likely to be associated with significant improvement in renal function.

The standard dosing regimen is designed to maintain eculizumab serum levels > 35 μg/mL, which is sufficient to completely and consistently block complement-mediated hemolysis in patients with PNH. In the multinational studies ($N = 195$), 100% of patients showed a strong response to the standard eculizumab dosing regimen as measured by a significant reduction in LDH [4, 21, 22]. In the current trial, two patients treated with eculizumab were not observed to show a rapid and strong reduction in LDH despite eculizumab serum concentration above 35 μg/mL. In these two patients, a partial drug response was observed with a reduction in complement activation as measured by

in vitro CH50 assays. This is an extremely rare event, perhaps unique to Japanese patients. Efforts are underway to examine the molecular linkage between CH50 and LDH in these two patients. Twenty-seven patients who each showed a strong response to treatment were enrolled in an extension trial and continued eculizumab treatment.

The review of safety parameters in this study shows that eculizumab, administered per the specified induction and maintenance dose, appears safe and well tolerated. Most AEs were mild (88%). There were no major adverse vascular events, thrombotic events, infusion reactions or episodes of anaphylaxis reported during the study. No patient discontinued participation in the study due to an AE or died. The safety profile of eculizumab reported in the Japanese trial was also consistent with that reported in the multinational phase III trials of eculizumab. The most frequent treatment-emergent AEs in trial were headache (52%), nasopharyngitis (41%), and nausea (21%), similar to the most common AEs in the SHEPHERD multinational study (headache 52.9%; nasopharyngitis 32%; upper respiratory tract infection 29.9%; nausea 20.6%) [4]. Furthermore, the observation is that most headaches were mild and the kinetics of the reported headaches in the Japanese trial was consistent with the observations in the SHEPHERD multinational study. Specifically, in SHEPHERD, 94% of patients who experienced headache did so within the first 48 h of drug administration and most were restricted to the first 2 weeks of therapy. A rapid increase in levels of nitric oxide has been shown to result in the transient induction of headache through vasodilatation [8], suggesting that headaches observed with eculizumab in the AEGIS and multinational PNH study populations may be related to the initial, rapid therapeutic reductions in intravascular hemolysis, cell-free Hgb, and nitric oxide consumption.

Thrombosis is the leading cause of death due to PNH in the Caucasian population, accounting for 40–67% of deaths [21]. However, past studies have suggested that TE is less prominent in Japanese patients with PNH [27, 28]. In the current study, we note that 5/29 or 17% of patients entering the Japanese PNH trial had a history of TE. This incidence is similar to the 19% observed in the randomized, double blind placebo PNH multinational phase III trial [22]. Previous studies have also demonstrated the ongoing TE risk in PNH patients, despite the absence of clinical evidence of TE [16]. Western patients with PNH have been shown to have subclinical TE (as detected by MRI) elevated levels of prothrombotic (e.g. D-dimer) and pro-inflammatory (e.g. IL-6) markers even without evidence of previous clinical thrombosis [13, 16, 40]. Consistent with these previous studies, we observed that 38% (11/29) of Japanese PNH patients had D-dimer levels above the ULN at baseline. Indeed, 52% (15/29) of Japanese patients were at demonstrably elevated risk for TE as indicated by either an elevated D-dimer measurement and/or history of documented thrombosis.

TE appears to be mediated by terminal complement activation in Japanese PNH patients. The observations that a significant proportion of Japanese PNH patients have elevated D-dimer levels at baseline and that eculizumab treatment normalizes this measure of hemostatic activation further confirm both the elevated risk for TE and the beneficial effect of eculizumab treatment in Japanese PNH patients. Indeed, the substantial reduction of TEs during eculizumab treatment in the multinational PNH study (over 281 patient years) empirically demonstrates that terminal complement activation plays a prominent role in the pathogenesis of thrombosis. There are strong similarities between Western and Japanese PNH patients. It has been clearly demonstrated that PNH evolves from the same somatic genetic mutations in the PIGA gene in both Western and Japanese populations [38, 41]. Additionally, the impact of blocking terminal complement-mediated hemolysis on significant morbidities and potentially life-threatening complications in Japanese patients is similar to that observed in the multinational trials, demonstrating that the physiology of hemolysis does not differ between the Japanese and Western population. It is possible that previously reported minor differences in patient presentation or symptoms may be related to different patterns of diagnosis of PNH or cultural sensitivities to various morbidities, low sample sizes due to rarity of the disease, or unappreciated comorbid factors in the two regions. In the future, participation in a PNH registry may be able to address cultural differences in regard to clinical manifestations in PNH patients.

Chronic eculizumab treatment provided clinically meaningful benefit to PNH patients in this study by reducing the primary manifestation of PNH, chronic intravascular hemolysis, with consequent significant improvements in fatigue, dyspnea, overall QoL, kidney disease, hemostatic activation and measures of thrombotic risk, anemia and transfusion requirements. These results demonstrate that eculizumab treatment provides significant clinical benefit to Japanese patients with PNH and the substantial reductions in morbidities and complications are consistent with the clinical benefits observed in the previous multinational studies.

References

1. Aaronson NK, Ahmedzai S, Bergman B, Bullinger M, Cull A, Duez NJ, Filiberti A, Flechtner H, Fleishman SB, de Haes JC. The European Organization for Research and Treatment of Cancer QLQ-C30: a quality-of-life instrument for use in

international clinical trials in oncology. J Natl Cancer Inst. 1993;85:365–76.
2. Audebert HJ, Planck J, Eisenburg M, Schrezenmeier H, Haberl RL. Cerebral ischemic infarction in paroxysmal nocturnal hemoglobinuria report of 2 cases and updated review of 7 previously published patients. J Neurol. 2005;252:1379–86.
3. Bessler M, Mason PJ, Hillmen P, Miyata T, Yamada N, Takeda J, Luzzatto L, Kinoshita T. Paroxysmal nocturnal haemoglobinuria (PNH) is caused by somatic mutations in the PIG-A gene. EMBO J. 1994;13:110–7.
4. Brodsky RA, Young NS, Antonioli E, Risitano AM, Schrezenmeier H, Schubert J, Gaya A, Coyle L, de Castro CC, Fu CL, Maciejewski JP, Bessler M, Kroon HA, Rother RP, Hillmen P. Multicenter phase 3 study of the complement inhibitor eculizumab for the treatment of patients with paroxysmal nocturnal hemoglobinuria. Blood. 2008;111:1840–7.
5. Cella D. Manual of the Functional Assessment of Chronic Illness Therapy (FACIT) measurement system. 4th ed. Evanston, IL: Center on Outcomes, Research and Education (CORE), Evanston Northwestern Healthcare and Northwestern University; 1997.
6. Cella D, et al. The FACIT-Fatigue Scale: description, reliability and validity. Evanston, IL: Center on Outcomes, Research and Education; 2003. p. 1–18.
7. Cella D, Lai JS, Chang CH, Peterman A, Slavin M. Fatigue in cancer patients compared with fatigue in the general United States population. Cancer. 2002;94:528–38.
8. Christiansen I, Iversen HK, Olesen J. Headache characteristics during the development of tolerance to nitrates: pathophysiological implications. Cephalalgia. 2000;20:437–44.
9. Clark DA, Butler SA, Braren V, Hartmann RC, Jenkins DE Jr. The kidneys in paroxysmal nocturnal hemoglobinuria. Blood. 1981;57:83–9.
10. de Latour RP, Mary JY, Salanoubat C, Terriou L, Etienne G, Mohty M, Roth S, de Guibert S, Maury S, Cahn JY, Socie G. Paroxysmal nocturnal hemoglobinuria: natural history of disease subcategories. Blood. 2008;112:3099–106.
11. Delles C, Klingbeil AU, Schneider MP, Handrock R, Schaufele T, Schmieder RE. The role of nitric oxide in the regulation of glomerular haemodynamics in humans. Nephrol Dial Transpl. 2004;19:1392–7.
12. Gabbai FB. Effects of nitric oxide synthase blockers on renal function. Nephrol Dial Transpl. 2001;16(Suppl 1):10–3.
13. Helley D, de Latour RP, Porcher R, Arrais C, Fauroux I, Matheron J, Duval A, Shved JF, Fischer AM, Socie G. Eculizumab inhibits clot generation and endothelial dysfunction in patients with paroxysmal nocturnal haemoglobinuria. Haematologica. 2010;95:574–81.
14. Hill A, Muus P, Duhrsen U, Socie G, Risitano A, De Paz R, Van den Neste E, Zanella A, Lai J, Hillmen P, Rother R, Cella D. Improvement in fatigue with eculizumab treatment of patients with PNH occurs independent of changes in anemia. EHA. 2008.
15. Hill A, Rother RP, Wang X, Morris SM, Quinn-Senger K, Richards SJ, Bessler M, Kelly R, Hillmen P, Gladwin M. Effect of eculizumab on haemolysis-associated nitric oxide depletion, dyspnoea, and measures of pulmonary hypertension in patients with paroxysmal nocturnal haemoglobinuria. Br J Haematol. 2010;149:414–25.
16. Hill A, Reid SA, Rother RP, Gladwin MT, Collinson PO, Gaze DC, Lowe A, Guthrie A, Sivananthan MU, Hillmen P. High definition contrast-enhanced mr imaging in paroxysmal nocturnal hemoglobinuria (PNH) suggests a high frequency of subclinical thrombosis. Blood. 2006;108(11) (Abstract 979).
17. Hill A, Hillmen P, Richards SJ, Elebute D, Marsh JC, Chan J, Mojcik CF, Rother RP. Sustained response and long-term safety of eculizumab in paroxysmal nocturnal hemoglobinuria. Blood. 2005;106:2559–65.
18. Hill A, Richards SJ, Hillmen P. Recent developments in the understanding and management of paroxysmal nocturnal haemoglobinuria. Br J Haematol. 2007;137:181–92.
19. Hillmen P, Elebute M, Kelly R, Urbano-Ispizua A, Rother R, Khursigara G, Fu CL, Browne P, Rosse W. Long-term effect of the complement inhibitor eculizumab on kidney function in patients with paroxysmal nocturnal hemoglobinuria. Am J Hematol. 2010;85(8):553–9.
20. Hillmen P, Lewis SM, Bessler M, Luzzatto L, Dacie JV. Natural history of paroxysmal nocturnal hemoglobinuria. N Engl J Med. 1995;333:1253–8.
21. Hillmen P, Muus P, Duhrsen U, Risitano AM, Schubert J, Luzzatto L, Schrezenmeier H, Szer J, Brodsky RA, Hill A, Socie G, Bessler M, Rollins SA, Bell L, Rother RP, Young NS. Effect of the complement inhibitor eculizumab on thromboembolism in patients with paroxysmal nocturnal hemoglobinuria. Blood. 2007;110:4123–8.
22. Hillmen P, Young NS, Schubert J, Brodsky RA, Socie G, Muus P, Roth A, Szer J, Elebute MO, Nakamura R, Browne P, Risitano AM, Hill A, Schrezenmeier H, Fu CL, Maciejewski J, Rollins SA, Mojcik CF, Rother RP, Luzzatto L. The complement inhibitor eculizumab in paroxysmal nocturnal hemoglobinuria. N Engl J Med. 2006;355:1233–43.
23. Kanakura Y, Ohyashiki K, Shichishima T, Okamoto S, Ando K, Ninomiya H, Kawaguchi T, Nakao S, Nakakuma H, Nishimura J, Kinoshita T, Bedrosian C, Valentine ME, Ozawa K, Omine M. Chronic renal insufficiency in japanese patients with paroxysmal nocturnal hemoglobinuria (PNH): improvement with eculizumab treatment in the long-term follow-up of the AEGIS Study. Blood. 2009;114 (Abstract 1980).
24. Muus P, Risitano A, Castro-Malaspina H, Jones C, Fuller S, Socie G. Clinical impact of unregulated terminal complement activity in never-transfused patients with paroxysmal nocturnal hemoglobinuria. Blood. 2009;114 (Abstract).
25. Nath KA, Vercellotti GM, Grande JP, Miyoshi H, Paya CV, Manivel JC, Haggard JJ, Croatt AJ, Payne WD, Alam J. Heme protein-induced chronic renal inflammation: suppressive effect of induced heme oxygenase-1. Kidney Int. 2001;59:106–17.
26. National Kidney Foundation. K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification, and stratification. 2002.
27. Nishimura J, Kanakura Y, Ware RE, Shichishima T, Nakakuma H, Ninomiya H, Decastro CM, Hall S, Kanamaru A, Sullivan KM, Mizoguchi H, Omine M, Kinoshita T, Rosse WF. Clinical course and flow cytometric analysis of paroxysmal nocturnal hemoglobinuria in the United States and Japan. Medicine (Baltimore). 2004;83:193–207.
28. Parker C, Omine M, Richards S, Nishimura J, Bessler M, Ware R, Hillmen P, Luzzatto L, Young N, Kinoshita T, Rosse W, Socie G. Diagnosis and management of paroxysmal nocturnal hemoglobinuria. Blood. 2005;106:3699–709.
29. Parker CJ. The pathophysiology of paroxysmal nocturnal hemoglobinuria. Exp Hematol. 2007;35:523–33.
30. Rinder CS, Rinder HM, Smith BR, Fitch JC, Smith MJ, Tracey JB, Matis LA, Squinto SP, Rollins SA. Blockade of C5a and C5b-9 generation inhibits leukocyte and platelet activation during extracorporeal circulation. J Clin Invest. 1995;96:1564–72.
31. Rosse W. Paroxysmal nocturnal hemoglobinuria. In: Hoffman R, editor. Hematology: basic principles and practice. 3rd ed. Philadelphia: Churchill Livingstone, Inc. 2000.
32. Rother RP, Bell L, Hillmen P, Gladwin MT. The clinical sequelae of intravascular hemolysis and extracellular plasma hemoglobin: a novel mechanism of human disease. JAMA. 2005;293:1653–62.
33. Rother RP, Rollins SA, Mojcik CF, Brodsky RA, Bell L. Discovery and development of the complement inhibitor eculizumab

for the treatment of paroxysmal nocturnal hemoglobinuria. Nat Biotechnol. 2007;25:1256–64.
34. Schlaich MP, Schmitt D, Ott C, Schmidt BM, Schmieder RE. Basal nitric oxide synthase activity is a major determinant of glomerular haemodynamics in humans. J Hypertens. 2008;26:110–6.
35. Schneider R, Raff U, Vornberger N, Schmidt M, Freund R, Reber M, Schramm L, Gambaryan S, Wanner C, Schmidt HH, Galle J. L-Arginine counteracts nitric oxide deficiency and improves the recovery phase of ischemic acute renal failure in rats. Kidney Int. 2003;64:216–25.
36. Socie G, Mary JY, de Gramont A, Rio B, Leporrier M, Rose C, Heudier P, Rochant H, Cahn JY, Gluckman E. Paroxysmal nocturnal haemoglobinuria: long-term follow-up and prognostic factors. French Society of Haematology. Lancet. 1996;348:573–7.
37. Socie G, Hillmen P, Muus P, Schubert J, Duhrsen U, Risitano AM, Rother RP, Brodsky RA, Szer J. Sustained improvements in transfusion requirements, fatigue and thrombosis with eculizumab treatment in paroxysmal nocturnal hemoglobinuria. ASH Annu Meet Abstr. 2007;110:3672.
38. Takeda J, Miyata T, Kawagoe K, Iida Y, Endo Y, Fujita T, Takahashi M, Kitani T, Kinoshita T. Deficiency of the GPI anchor caused by a somatic mutation of the PIG-A gene in paroxysmal nocturnal hemoglobinuria. Cell. 1993;73:703–11.
39. Thomas TC, Rollins SA, Rother RP, Giannoni MA, Hartman SL, Elliott EA, Nye SH, Matis LA, Squinto SP, Evans MJ. Inhibition of complement activity by humanized anti-C5 antibody and single-chain Fv. Mol Immunol. 1996;33:1389–401.
40. Weitz IC, Ghods M, Rochanda L, Prazavi P, Zwicker J, Furie B, Liebman H. Eculizumab therapy results in rapid and sustained decreases in markers of thrombin generation and inflammation in patients with PNH. ASH Annu Meet Abstr. 2008;112:407.
41. Yamada N, Miyata T, Maeda K, Kitani T, Takeda J, Kinoshita T. Somatic mutations of the PIG-A gene found in Japanese patients with paroxysmal nocturnal hemoglobinuria. Blood. 1995;85:885–92.

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ORIGINAL ARTICLE

GPI-anchored protein-deficient T cells in patients with aplastic anemia and low-risk myelodysplastic syndrome: implications for the immunopathophysiology of bone marrow failure

Takamasa Katagiri[1,2], Zhirong Qi[2], Shigeki Ohtake[1], Shinji Nakao[2]

[1]Clinical Laboratory Science, Division of Health Sciences; [2]Cellular Transplantation Biology, Division of Cancer Medicine, Kanazawa University Graduate School of Medical Science, Kanazawa, Ishikawa, Japan

Abstract

Glycosylphosphatidylinositol-anchored protein-deficient (GPI-AP$^-$) T cells can be detected in some patients with bone marrow failure (BMF), but the link between these cells and BMF pathophysiology remains to be elucidated. To clarify the significance of GPI-AP$^-$ T cells in BMF, peripheral blood from 562 patients was examined for the presence of CD48$^-$CD59$^-$CD3$^+$ cells using high-resolution flow cytometry (FCM), and the GPI-AP$^-$ T cells were characterized with regard to their phenotype and sensitivity to inhibitory molecules, including herpesvirus entry mediator (HVEM) and a myelosuppressive cytokine, TGF-β. A multi-lineage FCM analysis detected CD48$^-$CD59$^-$CD3$^+$ T cells in 72 (12.8%) of the patients, together with GPI-AP$^-$ myeloid cells. Unexpectedly, 12 patients (10 with aplastic anemia and 2 with myelodysplastic syndrome-refractory anemia, 2.1%), who showed clinical features similar to those of other BMF patients with GPI-AP$^-$ myeloid cells, such as a good response to immunosuppressive therapy, displayed 0.01–0.3% GPI-AP$^-$ cells exclusively in T cells. The CD48$^-$CD59$^-$ T cells consisted of predominantly effector memory (EM) and terminal effector cells, while CD48$^-$CD59$^-$ T cells from non-BMF patients who had received anti-CD52 antibody only showed EM and central memory phenotypes. TGF-β and HVEM capable of inhibiting T-cell proliferation via its GPI-AP CD160 ligation suppressed the *in vitro* proliferation of GPI-AP$^+$ T cells more potently than that of GPI-AP$^-$ T cells from the same patients. The presence of GPI-AP$^-$ T cells, as well as GPI-AP$^-$ myeloid cells, may therefore reflect the immunopathophysiology of BMF in which cytokine-mediated suppression of hematopoietic stem cells via GPI-AP-type receptors takes place.

Key words aplastic anemia; myelodysplastic syndrome; paroxysmal nocturnal hemoglobinuria; GPI-anchored protein-deficient T cells

Correspondence Shinji Nakao, Cellular Transplantation Biology, Division of Cancer Medicine, Kanazawa University Graduate School of Medical Science, 13-1 Takaramachi Kanazawa, Ishikawa, Japan. Tel: +81 762 652 274; Fax: +81 762 34 4252; e-mail:snakao@med3.m.kanazawa-u.ac.jp

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Small populations of glycosylphosphatidylinositol-anchored protein-deficient (GPI-AP$^-$) blood cells are often detectable in the peripheral blood (PB) of patients with aplastic anemia (AA) and low-risk myelodysplastic syndrome (MDS) such as refractory anemia (RA) and refractory cytopenia with multilineage dysplasia (1–6). Although such GPI-AP$^-$ blood cells often comprise $<1\%$ of granulocytes or erythrocytes, they are thought to be derived from hematopoietic stem cells (HSCs) with a *PIGA* mutation rather than committed progenitor cells because GPI-AP$^-$ granulocytes persists for many years, maintaining their individual scattergram profiles (7). Several studies have identified the presence of small populations of GPI-AP$^-$ cells as a significant factor predicting a good response to immunosuppressive therapy (IST) in patients with AA and low-risk MDS (4–6, 8–10).

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Immune mechanisms are therefore thought to be involved in the increase in the GPI-AP$^-$ cells in such bone marrow failure (BMF), though the exact mechanisms responsible for the increase in the GPI-AP$^-$ cells remain unknown.

The most widely accepted mechanism for clonal expansion of GPI-AP$^-$ cells in patients with BMF is the 'escape hypothesis', which states that the relative number of *PIGA* mutant HSCs increases by avoiding immunologic attacks by T cells or NK cells (11–17). Consistent with the escape hypothesis, GPI-AP deficient cells are usually detectable in many lineages of cells, including monocytes, lymphocytes and NK cells, in addition to granulocytes and erythrocytes, in patients with classical paroxysmal nocturnal hemoglobinuria (PNH) (18–21). However, the screening of multi-lineage PB cells from patients with BMF for the presence of GPI-AP$^-$ cells using high-sensitivity flow cytometry unexpectedly revealed a few patients who showed GPI-AP$^-$ cells only in T cells (unpublished observation). The presence of GPI-AP$^-$ T cells alone appeared to contradict the escape mechanism because T-cell precursors are not the target of immune system attack in BMF. Therefore, the multilineage analysis was extended to a larger number of patients to determine the significance of GPI-AP$^-$ T cells in patients with BMF. The phenotypic and functional analyses of such GPI-AP$^-$ T cells provided evidence that, just like GPI-AP$^-$ myeloid cells, GPI-AP$^-$ T cells reflect the immunopathophysiology of BMF, in which the cytokine-mediated suppression of HSCs via GPI-AP-type receptors takes place.

Patients and methods

Patients and healthy volunteers

The PBs of 562 patients with various types of cytopenias were examined for the presence of GPI-AP$^-$ cells using high-sensitivity flow cytometry. Their diagnoses included classic PNH in 13, AA in 348, and MDS-RA in 201. The subgroups of MDS were defined according to the FAB classification (22). The male-to-female ratio was 1 : 1.2 (255 : 307), and the median age was 56 yr (range: 1–95 yr). PB samples from three patients (one with acute myelogenous leukemia and two with AA) who were conditioned with alemtuzumab (Campath1-H), a humanized monoclonal antibody (mAb) specific to CD52, for allogeneic stem cell transplantation as well as from 57 healthy individuals were also examined for the presence of GPI-AP$^-$ cells in all lineages of cells. All patients and healthy individuals provided their informed consent before sampling. This study protocol was approved by the ethics committee of Kanazawa University Graduate School of Medical Science.

Monoclonal antibodies (mAbs)

mAbs used for multicolor flow cytometry were anti-CD59 labeled with FITC (P282E, IgG2a; Beckman Coulter, Miami, FL, USA), anti-CD59 labeled with PE (H19, IgG2a; BD Pharmingen, San Diego, CA, USA), anti-CD55 labeled with FITC (IA10, IgG2a; BD Pharmingen), anti-CD48 labeled with FITC (J4-57, IgG1; Beckman Coulter), anti-CD48 labeled with PE (156-4H9, IgG1; eBioscience, San Diego, CA, USA), anti-CD33 labeled with APC (D3HL60.251, IgG1; Beckman Coulter), anti-CD19 labeled with APC-Cy7 (SJ25C1, IgG1; BD Pharmingen), anti-CD335 labeled with PE (BAB281, IgG1; Beckman Coulter), anti-CD3 labeled with PerCP-Cy5.5 (SK7, IgG1; BD Pharmingen), anti-CD3 labeled with APC (UCHT1, IgG1; Beckman Coulter), anti-CD11b/Mac-1 labeled with PE (ICRF44, IgG1; BD Pharmingen), anti-glycophorin A labeled with PE (JC159, IgG1; Dako, Carpenteria, CA, USA). Phenotypic analysis of GPI-AP-deficient CD3$^+$ T lymphocyte was carried out by additional staining with mAbs specific to CD45RA labeled with PE (HI100, IgG2b; BD Pharmingen), CD62L labeled with APC (DREG-56, IgG1; BD Pharmingen), CD197 labeled with PE-Cy7 (3D12, IgG2a; BD Pharmingen), CD4 labeled with APC-Cy7 (RPA-T4, IgG1; BD Pharmingen), CD8 labeled with APC-Cy7 (SK1, IgG1; BD Pharmingen).

Flow cytometry for detecting GPI-AP$^-$ cells and determining GPI-AP$^-$ T-cell phenotype

Six lineages of blood cells including granulocytes, erythrocytes, monocytes, T cells, B cells and NK cells were subjected to high-sensitivity flow cytometry for detecting small populations of GPI-AP$^-$ cells. All blood samples were analysed within 24 h to avoid false-positive results because of cell damages. The staining with the each mAb in this study was performed according to the well-established lyse-stain protocol, previously described in detail (6, 23). Briefly, 3–5 mL of heparinized blood was drawn from the patients and healthy individuals. Erythrocytes were lysed in the lysis buffer containing NH_4Cl 8.26 g/L, $KHCO_3$ 1.0 g/L, and EDTA · E4Na· 0.037 g/L to detect GPI-AP$^-$ leukocytes. After washing with saline, 50 μL of the leukocyte suspension was incubated with FITC-labeled anti-CD55 and anti-CD59 mAbs for granulocytes or FITC-labeled anti-CD48 and anti-CD59 mAbs for monocytes, T cells, B cells and NK cells in combination with mAbs specific for lineage markers including PE-labeled CD11b for granulocytes, APC-labeled CD33 for monocytes, PerCP-Cy5.5-labeled CD3 for T cells, APC-Cy7-labeled CD19 for B cells and PE-labeled CD335 for NK cells. Fresh

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blood was diluted to 3% in phosphate-buffered saline (PBS), and then 50 μL was incubated with PE-labeled anti-glycophorin A and FITC-labeled anti-CD55 and anti-CD59 mAbs on ice for 30 min to detect GPI-AP$^-$ erythrocytes. Three-step gating excluded the debris and immature granulocytes that are frequently found in samples from patients with MDS. Step 1 involved the gating of granulocyte, lymphocyte or monocyte populations from the FSC–SSC scattergrams (R1). Step 2 involved the gating of the lineage marker^{bright} population on the lineage marker-SSC scattergram to exclude the lineage marker^{dim} cells that are features of either damaged cells or immature cells. Step 3 was the gating of R1 × R2 and the analysis of 10^6 cells on R1 × R2 scattergrams. The presence of ≥0.005% CD55$^-$CD59$^-$GP-A$^+$ erythrocytes, ≥0.003% CD55$^-$CD59$^-$CD11b$^+$ granulocytes, and ≥0.01% CD55$^-$CD59$^-$ CD33$^+$ monocytes, CD48$^-$CD59$^-$ CD3$^+$ T cells, CD48$^-$CD59$^-$CD19$^+$ B cells and CD48$^-$CD59$^-$ CD335$^+$ NK cells was defined as an abnormal increase (positive) based on the results obtained from 57 healthy individuals (6). When GPI-AP$^-$ cells were detected in only one lineage of cells or the percentages of GPI-AP$^-$ cells were <0.01%, then additional samples were tested, and the patients were judged to be PNH$^+$ when the examination results of the first and second samples were identical.

The phenotype of GPI-AP-deficient-CD3$^+$ T lymphocyte was determined using anti-CD45RA, anti-CD62L and anti-CCR7 mAbs and the percentages of four different T-cell subsets including naïve (CD45RA$^+$CD62L$^+$CCR7$^+$), central memory (CM) (CD45RA$^-$CD62L$^+$CCR7$^+$), effector memory (EM) (CD45RA$^-$CD62L$^-$CCR7$^-$), and terminal effector memory (TEM) (CD45RA$^+$CD62L$^-$ CCR7$^-$) cells were determined according to the methods defined by previous reports (24, 25).

Data acquisition was performed immediately after sampling using FACSCanto II, and the data were analysed using the FACSDiva software program and percentage of each population was calculated by FlowJo software 7.6.1 (Treestar, Ashland, OR, USA).

T cell culture

PB mononuclear cells (PBMCs) were isolated using density gradient centrifugation on Ficoll/Hypaque (Fresenius Kabi Norge AS, Halden, Norway). A sample of 1×10^6 PBMCs were cultured in RPMI1640 containing 10 μg/mL phytohemagglutinin (Sigma, St. Louis, MO), 10% fetal bovine serum (FBS), 50 U/mL penicillin, 50 μg/mL streptomycin and 100 IU/mL IL-2 for 7 d. After washing with RPMI1640, the cultured cells were subjected to cell sorting in order to analyse the *PIGA* gene as described in the following paragraphs.

Cell sorting and *PIGA* gene analysis

CD48$^-$CD59$^-$CD3$^+$ freshly isolated or cultured T cells were separated from CD3$^+$ T cells with a cell sorter (JSAN; Bay Bioscience, Kobe, Japan). More than 95% of the sorted cells were GPI-AP deficient. An analysis of the *PIGA* gene mutation was performed as described previously (26). Briefly, the coding regions of *PIGA* were amplified by nested or semi-nested PCR using 12 primer sets, and six ligation reactions were used to transform competent *Escherichia coli* JM109 cells (Nippon Gene, Tokyo, Japan). Five clones were selected randomly from each group of transfectants and subjected to sequencing with BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, San Diego, CA, USA) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Preparation of CD3$^+$ T cells and mAb-coated latex beads

CD3$^+$ T cells were purified from freshly isolated PBMCs by depleting non-CD3$^+$ T cells with magnetic beads using Pan T cell isolation II kit II (Miltenyi, Bergisch Gladbach, Germany); purity was judged to be over 95% by flow cytometry. Latex beads (Miltenyi) were coated with various concentration of anti-CD3 (OKT3; Miltenyi) and anti-CD28 mAbs (15E8; Miltenyi), or various concentrations of human herpesvirus entry mediator (HVEM)-Ig (mIgG1 Fc; 100-330; R&D Systems, Minneapolis, MN, USA) or mouse IgG1 (27). The mixture of latex beads were suspended in PBS and incubated for 2 h at 37°C in humid air containing 5% CO_2. The latex beads were washed once with RPMI1640 medium containing 10% FBS for 30 min at 37°C. The beads were then washed three times with PBS and thereafter were used for T-cell stimulation.

Carboxyfluorescein diacetate succinimidyl diester (CFSE) assay

CD3$^+$ T cells were washed twice with PBS and were suspended in PBS at the concentration of 5×10^6 cells/mL. One milliliter of the cell suspension was mixed with an equal volume of PBS containing 1 μM CFSE (Invitrogen, Carlsbad, CA, USA), and incubated for 10 min in a humidified atmosphere containing 5% CO_2 at 37°C with occasional mixing. Labeling was quenched by the additional of an equal volume of cold FBS, and incubated for 5 min on ice. The cells were then centrifuged and washed three times in PBS containing 1% bovine serum albumin followed by two washes with RPMI1640 containing 5% autologous serum. The cells were plated at a density of 1×10^6 cells/mL in 96-well U-bottomed plates and were incubated in the presence of anti-CD3 mAb-coated and anti-CD28 mAb-coated beads with or

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without HVEM fusion protein or TGF-β (Peprotech, Rocky Hill, NJ, USA) at various concentrations. The CFSE levels in the cultured T cells were then determined 10 d later by flow cytometry. The inhibitory effects of HVEM or TGF-β on the T-cell proliferation was assessed by comparing the mean percentage of cells that underwent cell division in the presence of the inhibitory molecules with that of the control culture.

Statistical analysis

The differences in the inhibition of the decline in the CFSE level by HVEM or TGF-β between GPI-AP$^+$ and GPI-AP$^-$ T cells of individual patients were assessed by the Student's *t*-test.

Results

GPI-AP$^-$ T cells in patients with BMF

Significant populations of GPI-AP$^-$ cells were detectable in at least one lineage of cells from 252 (44.8%) of 562 patients with BMF and CD48$^-$CD59$^-$CD3$^+$ T cells were detected in 72 (12.8%) of the patients. Clone sizes of GPI-AP$^-$ cells in different lineages of cells in patients with increased GPI-AP$^-$ cells are summarized in Table 1. The GPI-AP$^-$ cells were also detected in two or more lineages of cells including granulocytes or monocytes in 60 of the GPI-AP$^-$ T cell$^+$ patients (Fig. 1A–C). However, the remaining 12 (2.1%) patients showed GPI-AP$^-$ cells only in T cells (Fig. 1D). The similar percentages (0.01–0.3%) of GPI-AP$^-$ T cells were detectable in different samples obtained from the patients at intervals of 2–6 months (Fig. 2). Such GPI-AP$^-$ T cells >0.01% were undetectable in any of 57 healthy individuals and the other 490 patients with BMF. The clinical characteristics of the 12 patients who were provisionally referred to as 'PNH-T$^+$ patients' are summarized in Table 2. All these patients had predominant thrombocytopenia without any increase in the number of BM megakaryocytes, a common feature of BMF patients possessing small populations of GPI-AP$^-$ cells (7). Five patients (patients 1, 2, 3, 9, and 11) were red blood cell or platelet transfusion-dependent, and only patient 11 had been treated with IST (ATG) before the detection of GPI-AP$^-$ T cells. Three of the PNH-T$^+$ patients received IST (ATG + cyclosporine for patients 2 and 9, and cyclosporine alone for patient 12) after the GPI-AP$^-$ cell screening. All achieved a partial remission according to the response criteria described by Camitta (28) as described previously.

Phenotype of GPI-AP$^-$ T cells detected in patients with BMF

The functional phenotypes of GPI-AP$^-$ T cells in nine PNH-T$^+$ patients, defined by the expression of CD45RA, CD62L, and CCR7 were compared to those of GPI-AP$^-$ T cells detectable in three BM transplant recipients who were conditioned with alemtuzumab (group 1) or to those detectable in 12 patients who displayed GPI-AP$^-$ cells in all lineages of blood cells including GPI-AP$^-$ T cells that account for 0.02–41.2% of total T cells (group 2). As shown in Fig. 3A, the GPI-AP$^-$ T cells in three patients from group 1 predominantly showed EM (CD45RA$^-$CD62L$^-$CCR7$^-$, EM) and CM (CD45RA$^-$CD62L$^+$CCR7$^+$, CM) phenotypes. No naïve (CD45RA$^+$CD62L$^+$CCR7$^+$) T-cell subset was observed in this group. On the other hand, the T cells from group 2 patients mainly contained cells with the naïve phenotype with relatively small percentages of CM, EM, and TEM subsets (Fig. 3B). GPI-AP$^-$ T cells in PNH-T$^+$ (group 3) patients predominantly showed the EM phenotype with smaller percentages of naïve, CM, and TEM phenotypes (Fig. 3C), suggesting the phenotypic pattern of GPI-AP$^-$ T cells in group 3 patients to be more similar to that in group 2 patients than that in non-BMF patients treated with alemtuzumab.

Table 1 Clone size of GPI-AP$^-$ cells in different lineages of cells in patients with increased GPI-AP$^-$ cells

	PNH	AA		MDS-RA	
	Median % of GPI-AP$^-$ cells (range)	% of patients with GPI-AP$^-$ cells in all AA patients	Median % of GPI-AP$^-$ cells in AA patients with increased GPI-AP$^-$ cells (range)	% of patients with GPI-AP$^-$ cells in all RA patients	Median % of GPI-AP$^-$ cells in RA patients with increased GPI-AP$^-$ cells (range)
E	25.8 (3.8–95.6)	58.4	0.04 (0.005–48.7)	53.3	0.55 (0.005–6.4)
G	56.5 (1.2–98.1)	64.4	0.07 (0.003–37.9)	57.8	1.07 (0.003–17.4)
M	82.6 (6.1–94.2)	48.4	0.11 (0.01–82.0)	46.7	3.5 (0.01–32.2)
T	0.9 (0.01–41.2)	16.8	0.44 (0.01–6.9)	9.4	0.23 (0.01–6.6)
B	4.3 (0.8–38.0)	16.0	0.01 (0.01–13.0)	12.2	0.24 (0.01–5.1)
NK	41.7 (0.6–94.9)	15.2	0.01 (0.01–75.0)	14.4	0.55 (0.01–8.5)

E, erythrocytes; G, granulocytes; M, monocytes; T, T cells; B, B cells; NK, NK cells; PNH, paroxysmal nocturnal hemoglobinuria; AA, aplastic anemia; MDS-RA, myelodysplastic syndrome-refractory anemia; GPI-AP, glycosylphosphatidylinositol-anchored protein.

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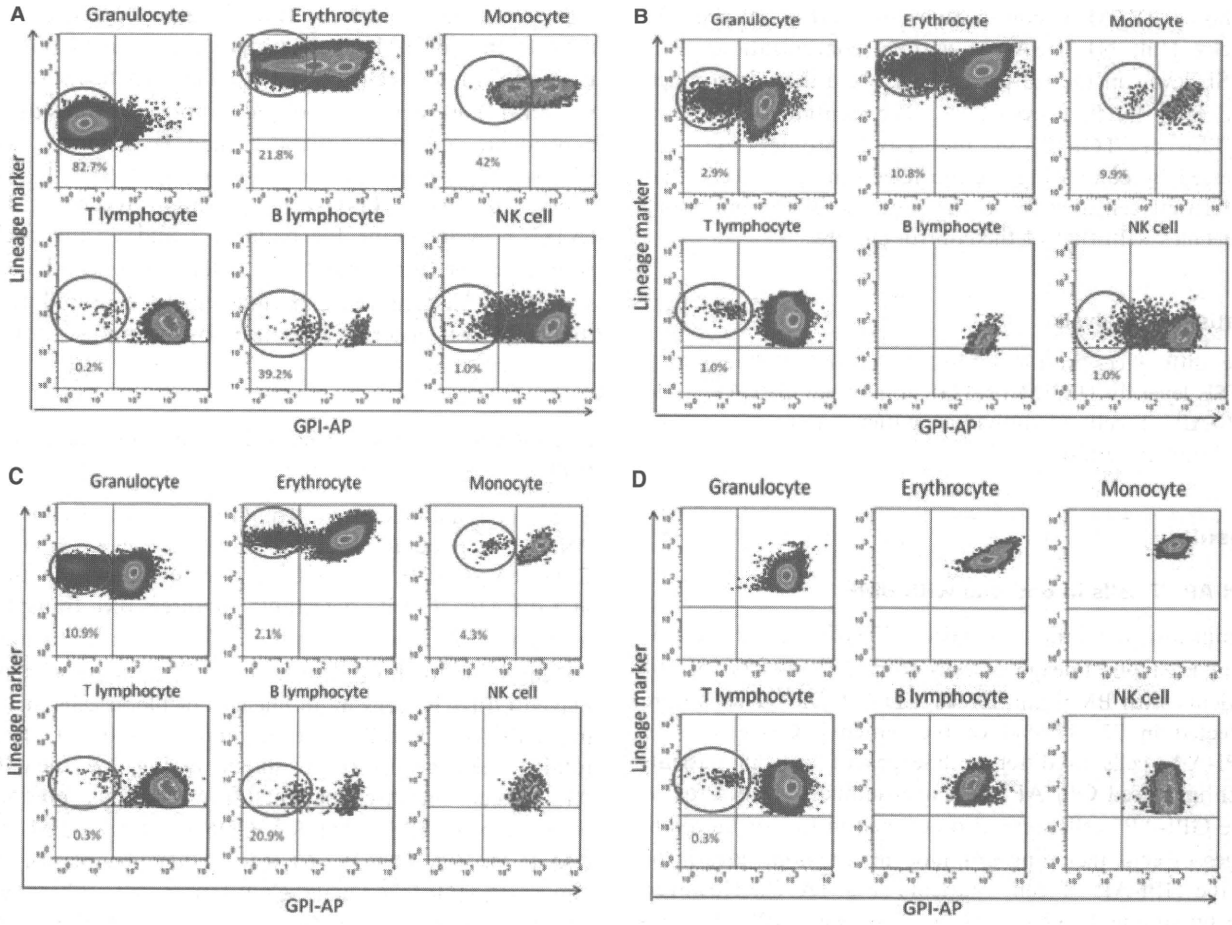

Figure 1 GPI-AP$^-$ cells in different lineages of cells. Scattergrams from four patients displaying GPI-AP$^-$ T cells are shown. (A) A patient with GPI-AP$^-$ cells in all six lineages of cells. (B) A patient with GPI-AP$^-$ cells in the granulocytes, erythrocytes, monocytes, T cells, and NK cells. (C) A patient with GPI-AP$^-$ cells in the granulocytes, erythrocytes, monocytes, T cells, and B cells. (D) A patient (patient 3) with GPI-AP$^-$ cells in T cells alone. GPI-AP, glycosylphosphatidylinositol-anchored protein.

PIGA gene analyses of GPI-AP$^-$ T cells

GPI-AP$^-$ T cells were sorted from cultured T cells from four patients (two group 1 and two group 2 patients) and were subjected to *PIGA* gene analyses. Missense mutations in exon 2 leading to a frameshift or an amino acid change were detected. These included a 16-bp deletion starting from the position of 1107 bp (frameshift), adenine insertion at the position of 1239 bp (frameshift) in group 2, the replacement of adenine (A) with guanine at 1108 bp (Arg → Gly) and 2002 bp (Gln → Arg) in a group 1 patient and the replacement of cytosine (C) with thymine (T) at 1044 bp (frameshift) and TCA insertion at 1103 bp (frameshift) in another group 1 patient.

Differences in the sensitivity to inhibitory molecules between GPI-AP$^+$ and GPI-AP$^-$ T cells

The presence of PNH-T$^+$ patients with BMF suggests that the lack of GPI-APs may confer a growth advantage to GPI-AP$^-$ T-cell precursors or memory T cells over the GPI-AP$^+$ counterparts. To test this hypothesis, T cells from three patients with BMF PNH displaying 1% and 41.2% GPI-AP$^-$ T cells were stimulated with anti-CD3 and anti-CD28 mAbs and the effects of HVEM, a ligand of a GPI-AP CD160 which inhibits T-cell proliferation upon ligation to HVEM (27), on the proliferation of GPI-AP$^-$ and GPI-AP$^+$ T cells were investigated using the CFSE assay. Figure 4 shows the results from one of the three patients which produced similar results. CD160 expression was induced on GPI-AP$^+$ T cells by anti-CD3 and anti-CD28 mAbs in a dose-dependent fashion, while the antigen was not induced on GPI-AP$^-$ T cells (Fig. 4A). Both the GPI-AP$^-$ and GPI-AP$^+$ T cells proliferated in response to anti-CD3 and anti-CD28 mAbs (Fig. 4B,C), though GPI-AP$^-$ T cells tended to show greater proliferation than GPI-AP$^+$ T cells in keeping with previous reports (29, 30). The addition of HVEM-IgG inhibited the decline in the CFSE level of GPI-AP$^+$ T cells more

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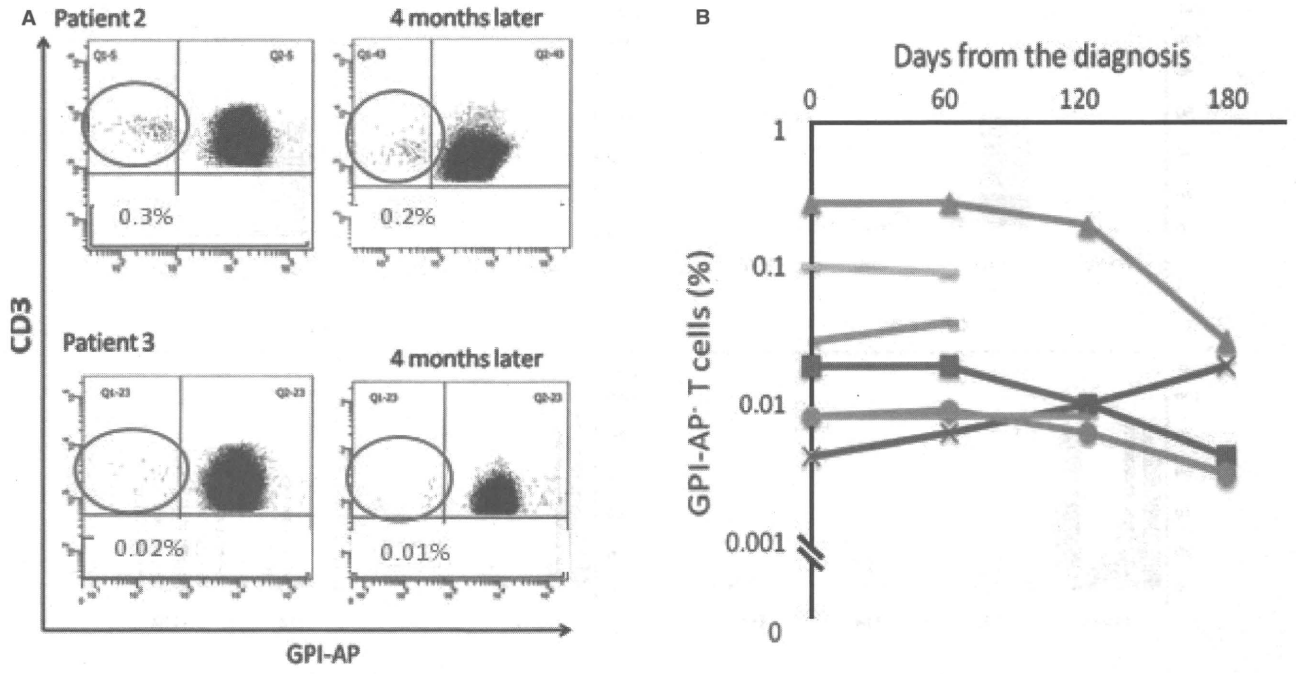

Figure 2 Changes in the percentage of GPI-AP$^-$ cells over time. (A) Scattergrams of samples obtained at 4 month internals from two PNH-T$^+$ patients are shown. (B) PB samples were obtained from the PNH-T$^+$ patients at 2-month intervals and the percentages of GPI-AP$^-$ T cells were serially determined. PNH, paroxysmal nocturnal hemoglobinuria; PB, peripheral blood; GPI-AP, glycosylphosphatidylinositol-anchored protein.

Table 2 Hematologic parameters of patients with GPI-AP$^-$ T cells alone

Patient no.	Diagnosis	Duration of illness (months)	Age at diagnosis (yr)	Gender	WBC count ($\times 10^9$/L)	Neutrophil count ($\times 10^9$/L)	Lymphocyte count ($\times 10^9$/L)	RBC count ($\times 10^{12}$/L)	Reticulocyte count ($\times 10^9$/L)	Hemoglobin (g/dL)	Platelet count ($\times 10^9$/L)	PNH-type T cell percentage
1	MAA	1	80	M	2.4	0.8	1.4	2.73	4.5	9.7	6.8	0.01
2	SAA	1	64	F	1.7	0.2	1.1	3.57	6.6	10.6	200	0.02
3	SAA	120	22	F	1.2	0.3	0.9	2.62	1.3	8.2	4	0.3
4	MAA	74	35	M	6.1	2.7	1.8	2.78	3.3	10.1	52	0.02
5	MAA	84	12	M	3.2	1	2	4.05	3.4	12.7	49	0.03
6	MDS-RA	4	59	M	5.7	2.9	2.7	2.06	5.6	10	53	0.02
7	SAA	12	61	F	1.2	0.4	0.6	2.52	0.8	7.6	12	0.01
8	MAA	108	83	F	2	1	0.8	3.51	3.2	9.8	64	0.03
9	MAA	49	26	F	2.6	0.9	1.2	2.22	4	7.3	0.8	0.1
10	MAA	120	59	M	2.1	0.8	1.2	1.56	2	5.9	0.9	0.03
11	MAA	1	76	M	2.7	1.1	1.5	4.03	4	12.4	8.2	0.3
12	MDS-RA	147	63	M	3.2	1.2	1.9	2.13	3.8	7.3	3	0.01

PNH, paroxysmal nocturnal hemoglobinuria; MDS-RA, myelodysplastic syndrome-refractory anemia; GPI-AP, glycosylphosphatidylinositol-anchored protein.

potently (27, 31, 32) than that of GPI-AP$^-$ T cells (Fig. 4B, 19.4 ± 8.1 vs. 0.7 ± 0.5 at 10 μg/mL, $P = 0.03$ and 57.5 ± 12.7 vs. 4.4 ± 4.9 at 50 μg/mL, $P = 0.004$). The GPI-AP$^-$ T-cell proliferation was not affected, even by a high concentration (50 μg/mL) of HVEM, which clearly inhibited GPI-AP$^+$ T-cell proliferation. Similarly, the addition of TGF-β tended to show a greater inhibition of the decline in the CFSE level of GPI-AP$^+$ T cells than that of GPI-AP$^-$ T cells (Fig. 4C, 17.4 ± 10.5 vs. 1.1 ± 0.5 at 5 ng/mL, $P = 0.06$ and 33.2 ± 16.2 vs. 2.1 ± 1.8 at 100 ng/mL, $P = 0.05$). These findings suggest that GPI-AP$^-$ CD3$^+$ T-cell precursors or memory T cells may preferentially proliferate *in vivo* in the presence of some ligands that transmit inhibitory signals for cell proliferation by their ligation to GPI-AP receptors.

Discussion

The present multi-lineage analysis of blood cells with high-sensitivity flow cytometry revealed CD48$^-$CD59$^-$ T

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