Brief report

Perforin gene mutations in patients with acquired aplastic anemia

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Perforin is a cytolytic protein expressed mainly in activated cytotoxic lymphocytes and natural killer cells. Inherited perforin mutations account for 20% to 40% of familial hemophagocytic lymphohistiocytosis, a fatal disease of early childhood characterized by the absence of functional perforin. Aplastic anemia, the paradigm of immune-mediated bone marrow failure syndromes, is characterized by hematopoietic stem cell destruction by activated T cells and Th1 cytokines. We examined whether mutations in the perforin gene occurred in acquired aplastic anemia. Three nonsynonymous *PRF1* mutations among 5 unrelated patients were observed. Four of 5 patients with the mutations showed some hemophagocytosis in the bone marrow at diagnosis. Perforin protein levels in these patients were very low or absent, and perforin granules were completely absent. Natural killer (NK) cell cytotoxicity from these patients was significantly decreased. Our data suggest that *PRF1* genetic alterations help explain the aberrant proliferation and activation of cytotoxic T cells and may represent genetic risk factors for bone marrow failure. (Blood. 2007;109: 5234-5237)

Introduction

Aplastic anemia is characterized by peripheral blood pancytopenia and a hypocellular bone marrow.[1] In most cases, aplastic anemia is an immune-mediated disease with active destruction of hematopoietic cells by activated cytotoxic T lymphocytes (CTLs) and increased IFN-γ levels[2]; natural killer (NK) cell numbers and NK cytolytic activity in vitro are decreased.[3]

NK cells and CTL can lyse tumor cells and virus-infected cells, but they also have activity against some normal cells that present self-antigens (such as immature dendritic cells), primarily to prevent autoimmunity. One of the pathways that NK cells and CTLs use to destroy target cells is release of cytolytic proteins. Perforin, a key component of this cytolytic process, is expressed mainly in CTLs and NK cells[4,5]; perforin is stored in cytoplasmic granules and is essential for killing by non–Fas-mediated mechanisms. Functional perforin is essential for normal CTL and NK cell function.[6-9]

We describe 4 unrelated patients with a mutation in exon 2 of *PRF1* gene and 1 patient with a mutation in exon 3; all mutations were in the coding region of *PRF1* and all but one gene carried also a polymorphism in exon 3. We hypothesize that genetic alterations in *PRF1* gene may contribute to the development of acquired aplastic anemia.

Patients, materials, and methods

Patients and controls

Informed consent was acquired according to protocols approved by the Institutional Review Board of the National Heart, Lung, and Blood Institute for all patient samples.

DNA from 75 unrelated patients with acquired aplastic anemia were sequenced for PRF1. For controls, resequence analysis was performed in both the SNP500 Cancer set and the Human Genomic Diversity Panel (HGDP)[10,11] (Document S1, available on the *Blood* website; see the Supplemental Document link at the top of the online article).

Nucleotide sequencing

Sequencing in DNA samples extracted from peripheral blood and buccal smear cells from patients and healthy controls was performed as previously described[12] (Document S1).

Western blot analysis, confocal microscopy, and cytotoxicity

Immunoblots were performed as previously described[2] (Document S1). Perforin granules in $CD8^+$ T cells were examined by confocal laser microscopy as previously described[13] (Document S1). Cytotoxic activity from patients' NK cells was compared with that from healthy controls as previously described.[13]

Statistical analysis

Differences in the frequencies of coding-sequence variations between samples from patients and those from healthy controls were evaluated by chi-square test using the Prism software (Prism Software, Irvine, CA).

Results and discussion

Patients with acquired aplastic anemia and perforin mutations

Of the 75 patients with acquired aplastic anemia examined, 3 novel, nonsynonymous mutations were identified in *PRF1* in 5 patients

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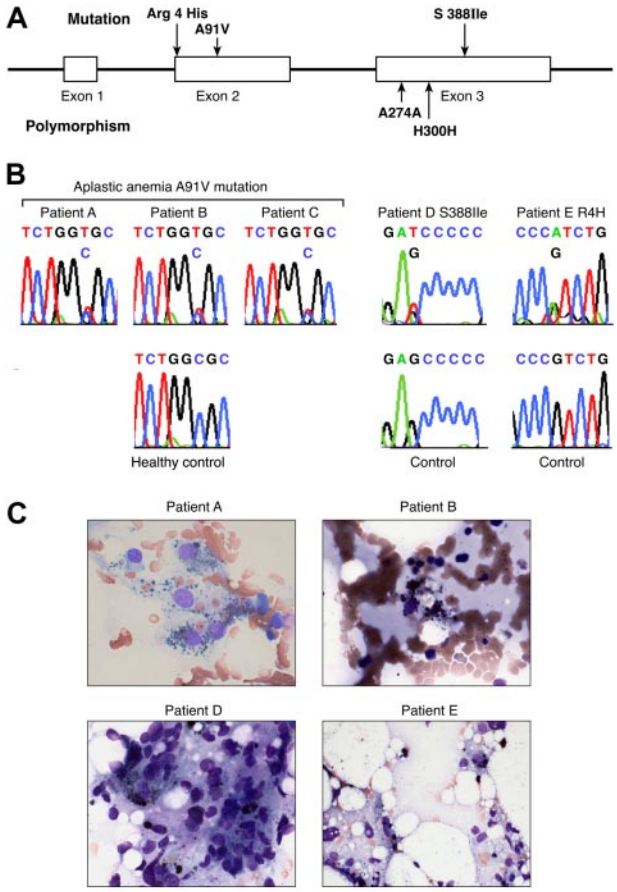

Figure 1. Perforin mutations in patients with aplastic anemia. (A) Linear structure of the *PRF1* gene, which encodes the human perforin protein and aplastic anemia-associated mutations. The segments represent the exons. *PRF1* has 3 exons, 2 of which (exons 2 and 3) contain coding sequences. In aplastic anemia, nonsynonymous mutations were found in exon 2 (codon 4 Arg/His and codon 91 Ala/Val) and exon 3 (codon 388 Ser/Ile). The polymorphisms in exon 3 are also shown (codons 274 and 300). R indicate arginine; H, histidine; I, isoleucine; V, valine; A, alanine. (B) Sequences of the patients carrying the mutations compared with sequences obtained from the controls. Green identifies adenine (A); red identifies thymidine (T); black identifies guanine (G); and blue identifies cytosine (C). (C) Bone marrow smear examination from the patients carrying *PRF1* mutations. Four of 5 patients carrying mutations revealed hemophagocytosis in bone marrow at diagnosis.

(Document S1; Figure 1; Tables 1-2). In 3 patients, *PRF1* sequencing revealed a heterozygous mutation in exon 2 in codon 91, with alanine replaced by valine (A91V). A previously known, common single nucleotide polymorphism in exon 3 (H300H) was also present. A novel mutation in exon 3 was identified in patient D: in codon 388, serine was replaced by isoleucine (S388I); this

Table 1. Screening for *PRF1* gene mutations in patients with aplastic anemia and healthy controls

Location of variation	Patients with aplastic anemia n = 75	Healthy controls n = 1156	*P*
Exon 2, codon 4 CGT/CAT (Arg/His),			
No. of patients (% of allele frequency)	1 (0.66)	21 (0.96)	.7
Exon 2, codon 91 GCG/GTG (Ala/Val)			
No. of patients (% of allele frequency)	3 (2)	24 (1.1)	.27
Exon 3, codon 388 AGC/ATC (Ser/Ile)			
No. of patients (% of allele frequency)	1 (0.66)	0 (0)	.001

The controls included 1036 from the HGDP, 102 from the SNP500 Cancer set, and 18 anonymous healthy persons.

Table 2. Clinical characteristics and laboratory profiles of patients carrying the *PRF1* mutations

Patient	Mutation	Age, y	Race or ethnic group*	Hemoglobin level, g/dL	Absolute neutrophil count, $\times 10^{-3}/mm^3$	Platelet count, $10^{-3}/mm^3$	Bone marrow cellularity, %	Hemophagocytosis	Follow-up
A	A91V/H300H	31	White	8.0	25	5000	5	Yes	No response to immunosuppression
B	A91V/H300H	77	White	8.8	95	11000	15	Yes	Relapse; CsA dependent
C	A91V/H300H	78	White	10.0	940	7000	10	No	Transient response; relapse; CsA dependent
D	S388I/A274A	33	Hispanic	5.8	950	9000	10	Yes	No response to immunosuppression
E	R4H	21	African American	4.1	780	6000	5	Yes	No response to immunosuppression

The peripheral blood and bone marrow counts at diagnosis (Document SI) are shown. All patients were men with a diagnosis of severe aplastic anemic. CsA indicates cyclosporine A. *Race or ethnic group was self-reported by the patients.

patient also carried a single nucleotide polymorphism in exon 3 (A274A). In patient E, a heterozygous mutation in exon 2 was identified: in codon 4, an arginine residue was replaced by histidine (R4H). The germ line origin of *PRF1* mutations was established by their detection in DNA extracted from buccal mucosa specimens obtained from 4 of 4 patients (A, B, C, and D).

In the normal immune response, antigen-presenting cells (APCs) can be eliminated through perforin-dependent cytotoxicity. The pathophysiology of familial hemophagocytic lymphohistiocytosis (FHLH), a fatal disease of the early childhood, is explained by the absence of perforin.[7,8] T cells receive activation signals through the APCs, but the APCs and the activated T cells are not eliminated, resulting in uncontrolled expansion of CTLs and CD34 destruction.[13] Perforin gene mutations account for 20% to 40% of FHLH cases linked to 10q21-22 (FHLH2).[14] Perforin consists of a leader and a lytic peptide, 2 regions of low homology, a conserved amphipathic α-helix, an EGF-like domain, a C2 domain, and a cleavable C terminus. Mutations have been identified in all domains but the cleavable C terminus[9]; approximately 48 different mutations in *PRF1* have been identified in FHLH2.[15] In idiopathic aplastic anemia, T cells are activated,[1,2,16] and here we examined whether perforin was involved in this activated T-cell phenotype.

The A91V mutation in exon 2 has been argued to be functionally important despite that it has also been reported to be a polymorphism in the general population, with an allele frequency ranging between 3% and 17%.[17-20] Our analysis of the A91V site in 52 worldwide populations with more than 1000 unrelated persons of diverse backgrounds revealed an overall prevalence of approximately 1% (we did not see an enrichment or increased prevalence in a particular geographic region or ethnic group). To our knowledge, we describe for the first time *PRF1* gene mutations in adults older than 30 years of age who had developed a significant hematologic disease. A communication describes atypical presentation of FHLH in 2 siblings in their mid-20s carrying the A91V mutation.[21] The A91V mutation may have a milder phenotype and accounting for later onset of clinical manifestations but, nevertheless, participate in the development of catastrophic immune-mediated syndromes.[20,22] Of note, alanine at position 91 is conserved among human, mouse, and rat perforin protein.[9]

Although the number of patients in our study is too small to draw a definitive conclusion, 4 of 5 patients with aplastic anemia with *PRF1* mutations showed evidence for hemophagocytosis in the bone marrow when first diagnosed, but there were no other typical clinical features of hemophagocytic syndrome[9,15,23,24] (Figure 1C). Historically, some elements of hemophagocytosis in bone marrow aspirates in severe aplastic anemia has long been recognized but may be underdescribed because of the dramatic empty marrow appearance that is pathognomonic of the disease.[25] Possibly *PRF1* gene mutations are related to a more severe phenotype of marrow failure, for which hemophagocytosis may be a morphologic marker of prognosis, because none of our patients with *PRF1* mutations experienced hematologic recovery with immunosuppressive treatment.

Perforin protein levels and cytolytic activity

PRF1 mutations in patients with aplastic anemia appear to be functionally important because they associate with low perforin protein levels and impaired cytolytic activity. Perforin protein levels were markedly reduced or absent in all patients carrying the *PRF1* mutations (n = 5) compared with the T cells of healthy controls (n = 8; Figure 2A). All but one of the patients with aplastic

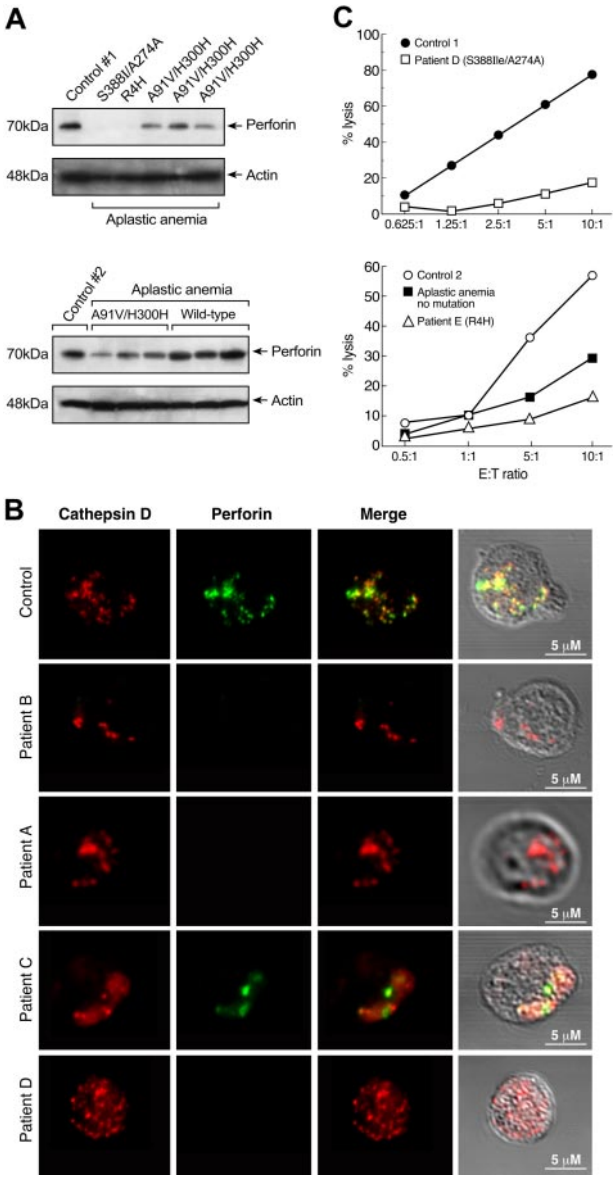

Figure 2. Decreased perforin protein levels and cytolytic activity in patients carrying *PRF1* mutations. (A) Cytoplasmic extracts from T cells of patients with aplastic anemia with *PRF1* mutations (n = 5) and healthy controls (n = 8) were analyzed for perforin protein expression by immunoblot. All 5 patients carrying *PRF1* mutations revealed decreased or absent perforin protein levels. All samples were run side by side. The upper panel (from left to right) shows the perforin protein levels from a healthy person and patients D, E, A, B, and C. The lower panel shows the immunoblot results from chronologically different samples obtained from the same patients (from left to right a healthy control, patient C, patient A, and patient B, and 3 patients with no mutations). Patients without *PRF1* mutations revealed perforin protein levels comparable to that from healthy volunteers. (B) Decreased perforin granules in cytotoxic cells from patients with aplastic anemia with *PRF1* mutations. $CD8^+$ T cells from patients carrying *PRF1* mutations and healthy controls were double stained in parallel with anti-cathepsin D (red, first column) and anti-perforin (green, second column) antibodies in at least 3 different experiments. Perforin and cathepsin D showed the expected pattern of colocalization in cytotoxic granules of control cells. Staining of the patients' cytotoxic cells revealed complete absence of perforin granules in patients A, B, and D (Figure 4); patient C showed slightly decreased staining of perforin granules, but they were abnormal in size. (Differences between perforin protein levels and perforin granule staining in patients A, B, and C, who have the same *PRF1* mutation may be explained by different genetic background or additional unshared genetic alterations). The frequency of cathepsin-D in $CD8^+$ T cells was similar in patients' and controls' samples. (C) Decreased cytolytic activity in patients with aplastic anemia. Natural killer cells were isolated from patients D and E, and their killing efficiency was determined in a standard Cr^{51}-release assay against K562 target cells in comparison to cells from healthy donors. Patients carrying *PRF1* mutations showed markedly decreased cytolytic activity compared with controls. A patient (not carrying any mutation or polymorphism) also showed decreased cytolytic activity but not as low as that observed in the patients with *PRF1* mutations.

anemia without *PRF1* mutations (n = 7) had similar amounts of perforin protein compared with healthy controls. Three patients with myelodysplastic syndrome and 2 patients with pure red cell aplasia, who served as disease controls, showed perforin protein levels comparable to those from healthy controls (data not shown).

To confirm the absence of perforin protein in patients carrying *PRF1* mutations, we examined cultured $CD8^+$ T cells from patients A, B, C, and D in comparison to healthy controls (n = 5) (sequencing for *PRF1* in these controls did not reveal mutations or polymorphisms) by confocal microscopy. Staining of the patients' cytotoxic cells revealed complete absence of perforin granules in patients A, B, and D (Figure 2B); patient C showed slightly decreased staining of perforin granules (Figure 2B). Patients with *PRF1* mutations also showed markedly decreased cytolytic activity (Figure 2C; aplastic anemia patients not carrying mutations (n = 2) also showed decreased cytolytic activity,[3] although not as low as observed in the patients with *PRF1* mutations).

Mechanistically, *PRF1* gene mutations may help explain the aberrant proliferation and activation of CTLs. Additional genetic variations in genes implicated in the homeostasis of the immune system and the down-regulation of an immune response cannot be excluded.[1] Here, we show that mutations in an immune regulatory mechanism previously identified in young children can manifest in adults without typically associated clinical findings or a suggestive family history, providing a further link between constitutional and acquired bone marrow failure syndromes.

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Authorship

Contribution: E.E.S. designed research, performed research, analyzed data, and wrote the paper; F.G. performed research and analyzed data; B.S. performed research and analyzed data; D.M. collected and analyzed data; M.B. performed research and analyzed data; V.V. performed research; S.G. provided technical support; R.C. analyzed data and revised the paper; S.J.C. designed research, analyzed data, and revised the paper; N.S.Y. designed research and wrote the paper.

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