Regular Article

Mosaicism of NK cells in a patient with Wiskott-Aldrich syndrome

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MIL performed the experiments; MIL and ERO designed the study and wrote the manuscript. DSB and FSR established the diagnosis and interpreted the patient’s disease.

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Abstract

Rare cases of somatic mosaicism resulting from reversion of inherited mutations can lead to attenuation of blood cell disorders including Wiskott-Aldrich syndrome (WAS). The impact of the revertant hematopoietic stem or progenitor cells, particularly their representation in blood cell populations, is of interest as it predicts the outcome of gene therapy. Here we report an 8-year-old patient with WAS caused by a single nucleotide insertion in the WASP gene that abrogates protein expression. The patient nonetheless presented with mild disease. We found reversion of the mutation in a fraction of patient lymphocytes. Forty percent of natural killer (NK) cells expressed WASP, and NK cells contained both mutated and revertant (normal) sequences. WASP was not expressed in patient T- or B-cells; T cells contained only the mutated sequence. The selective advantage of WASP+ NK cells was demonstrated also for carrier females. The enrichment of WASP+ revertant NK cells indicates that WASP provides selective advantage in this lineage and predicts the success of gene therapy for reconstituting the NK cell compartment. The importance of reconstituting the NK cell lineage is discussed.
Wiskott-Aldrich syndrome (WAS) is an X-linked disease characterized by thrombocytopenia, small platelets, eczema and immunodeficiency. The responsible gene encodes WASP, which functions in actin remodeling during activation of blood cells. WASP is decreased or absent in blood cells of WAS patients. Absence of WASP is associated with more severe disease including higher frequency of infections, autoimmune disease and malignancies.

Several recent reports describe WAS patients with spontaneous reversion of mutations or second site mutations that restore function. These rare reversions/corrections, which can be studied at the DNA and protein level, become evident if the corrected cells have a selective advantage leading to increased frequency in peripheral blood cells. Although reversions/corrections have been found both (and only) in T and B cell lineages, only T cells demonstrate selective advantage. The frequency of corrected cells was highest for memory T cells, suggesting that selection results from an advantage in proliferation and/or activation. These studies left unanswered the identity of the reverted progenitor cell and whether reversion, if it were to occur in a multipotent hematopoietic progenitor, would confer proliferative advantage outside the T cell lineage.

Here we report a novel case of somatic mosaicism resulting from spontaneous reversion of an inherited WASP mutation. The revertant sequence was confined to the patient’s NK cells, which were a mixture of WASP-negative and WASP-expressing cells and contained the inherited and the revertant gene sequences. Enrichment of WASP-positive cells arising from a single or few revertant NK progenitor cells indicates that WASP confers proliferative advantage to the NK cell compartment and predicts success of gene therapy in restoring this compartment.

Study design

Patients
The patient (P1) was evaluated as an infant in 1994 because of thrombocytopenia (platelet counts 20-50,000/µL) and very mild eczema. He did not suffer from recurrent infections. A diagnosis of X-linked thrombocytopenia was considered. Bone marrow aspirate revealed normal trilineage hematopoiesis. Peripheral blood immunophenotype at age 8 showed a decrease in T cells (22% CD4; 11% CD8) and an increase in NK cells (45%, CD16+CD56+). After the birth of a brother (P2) with thrombocytopenia and eczema, both boys were re-evaluated and diagnosed with WAS based on thrombocytopenia (<70,000/µL) and small platelet size (P1, 1.89µ; P2, 1.81µ; normal platelet size, 2.2 ± 0.12µ) . Diagnosis was confirmed by identifying the mutation as described.

Cell, DNA and protein analysis
Blood was collected in acid-citrate-dextrose under protocols approved by the Institutional Review Board including informed consent and was fractionated immediately or after overnight shipment at ambient temperature. Peripheral blood mononuclear cells (PBMC) were isolated by Histopaque-1077
centrifugation. NK cells were isolated by positive selection with CD56 monoclonal antibody (MoAb) (Clone N901, Beckman Coulter) and secondary antibody linked magnetic beads (Dynal, Lake Success, NY). T-cells were isolated using T-cell Negative Selection kit (Dynal). DNA was isolated using QiaAmp kits (Qiagen) and cloned into pSTBlue-1 vector (Novagen).

Western blots were stained with rabbit anti-WASP (W485) 8. For flow cytometry, blood samples (100 µL) were incubated with fluorescein isothiocyanate (FITC) CD3 (clone UCHT1), phycoerythrin (PE)-Cy5 CD56 (clone N901) and PE-Cy5 CD19 (clone J4.119) MoAbs (Beckmann Coulter) for 15 minutes at ~22°C. Erythrocytes were lysed with FACSTM Lysing Solution (BD Biosciences, Palo Alto, CA). Intracellular WASP staining was done with 5 µg/mL of PE-labeled B9 or isotype control MoAb (Santa Cruz) using Cytofix/Cytoperm kit (Pharmingen, Palo Alto, CA). Cells were analyzed immediately on the FACS-Calibur (Beckton Dickinson) by collecting 10,000 cells/sample.

**Results**

**Inherited Mutation**

Sequencing of whole blood genomic DNA of both brothers identified the insertion of an adenine in a run of six adenines in exon 4 (476-77insA; shown below). The mutation encodes a frameshift and results in WASP-negative cells.

**Protein expression**

Western blots revealed normal size WASP in PBMC of the patient (P1; Figure 1A). Detection by a C-terminal antibody indicates that the protein has an in-frame C-terminus. Protein expression level was 34% of normal. No WASP was detected in PBMC of the patient’s brother (P2, Figure 1A). These findings suggested a correcting mutation in cells of patient P1.

![Figure 1](image-url). Western blot and WASP gene analysis. (A). Western blot. Shown are PBMC of a normal healthy individual (N) and patients P2 and P1 stained with rabbit antibodies to WASP C-terminal 15 amino acids 8 or GAPDH. (B) Genomic DNA analysis. Shown are amplified WASP exon 4 regions of T cells and NK cells of patient (P1) and two representative clones of his NK cell DNA. Only the inherited 7 adenines was found for NK and T cells of the patient’s brother P2 (not shown). Only the 6 adenine sequence was found for normal NK and T cells (not shown).
Gene sequences

Genomic DNA was sequenced from isolated cell populations. Contrary to expectations, patient T cell DNA had only the inherited sequence with the abnormal run of 7 adenines (Figure 1B). T cell clones ($n = 8$) also had only the inherited mutation (not shown). In contrast, DNA of patient NK cells showed a mixture of two sequences, the predominant inherited sequence and a de novo sequence (Figure 1B). Amplification and cloning of NK cell DNA identified the de novo sequence as the revertant sequence with a normal run of six adenines. The revertant sequence was found in 3 of 10 clones and the remainder had the inherited mutation.

WASP in blood cells

Intracellular WASP staining was performed on the final blood specimens from the patient. Total lymphocytes and T, B and NK cells of normal healthy individuals expressed WASP, and the same cell types from the patient’s brother P2 showed no detectable WASP (Figure 2A). In contrast, total lymphocytes of patient P1 showed both WASP$\text{dim}$ and WASP$\text{bright}$ cells, suggesting somatic mosaicism. On subset analysis, WASP-expressing cells were present only among patient NK cells (CD3$^+$ CD56$^+$), where they amounted to 40-48%. Patient T (CD3$^+$) and B lymphocytes (CD19$^+$) (Figure 2A) were WASP-negative. Among patient NK cells, WASP-expressing cells were frequent among CD56$\text{dim}$ cells (48%), whereas only 3% of CD56$\text{bright}$ cells were WASP-positive (Figure 2B).

Figure 2. Flow cytometric analysis. Cells in whole blood were stained for surface antigens, fixed and permeabilized and stained with PE-labeled WASP (blue) or isotype control (red) MoAbs. (A) Shown are total lymphocytes, T lymphocytes (CD3$^+$), B lymphocytes (CD19$^+$) and NK cells (CD3$^+$ CD56$^+$) of a normal individual (N), the revertant patient (P1) and his brother (P2). (B) Contour plot of patient (P1) NK cells stained with CD3 and CD56 and histograms showing WASP staining of CD56$\text{bright}$ and CD56$\text{dim}$ subpopulations. (C) Analysis of T lymphocytes, B lymphocytes, NK cells, and NK subsets of an (age-matched) normal female and a female WAS carrier (large deletion of WASP gene). Note the presence among cells of the female carrier of ~88% WASP$^+$ CD56$\text{dim}$ cells, but only 78% WASP$^+$ CD56$\text{bright}$ cells. Similar findings were obtained for a second carrier female (intron 6 $+5g\rightarrow a$), 90% WASP$^+$CD56$\text{dim}$ NK cells, 78% of WASP$^+$ CD56$\text{bright}$ cells.
We also examined NK cells of female carriers of WAS as an independent measure of the selective advantage conferred by WASP expression. A non-random pattern of X-chromosome inactivation has been demonstrated for mononuclear cells of carriers of WAS (reviewed in 9). On analysis of WASP expression in the female carrier, selective advantage for WASP⁺ NK cells was demonstrated by the ~90% WASP⁺NK cells (Figure 2C). Consistent with the findings for the revertant patient, the percent of WASP⁺ cells was greater for the CD56dim population than the CD56bright. In contrast, T and B cells were 100% WASP⁺. Similar findings were obtained for a second carrier female.

Discussion

Since WASP functions in dynamic cytoskeletal rearrangements, the enrichment of the patient’s revertant NK cells might be due to selective advantage of WASP⁺ vs. WASP⁻ progenitor or precursor cells in migratory capacity, chemokine responsiveness, proliferative responsiveness, and/or survival capacity. The greater frequency of revertant WASP⁺ cells among the CD56dim subset of terminally differentiated NK cells relative to CD56bright subset suggests that part of the selective advantage is at the level of peripheral cells as noted also for memory vs. naïve T cells of previous revertant patients. CD56dim cells express a range of activating and inhibitory receptors and homing receptors for inflammatory tissue sites; they are perforin-positive and display high cytotoxicity 10. The majority of CD56bright NK cells reside in secondary lymphoid organs; they are most potent cytokine producers and acquire phenotype similar to peripheral NK cells and ability to lyse target cells on stimulation with interleukin-2 in vitro 11,12,13. It is possible that the infrequency of revertants in this subset is due in part to revertant WASP expressing CD56bright NK cells successfully leaving the circulation and homing to secondary lymphoid organs.

Vital functions of NK cells include anti-viral and anti-tumor cytolytic activity and an immunoregulatory role in which NK cells and dendritic cells interact to provide innate protection against pathogens and generate protective adaptive immune responses 14-17. WASP plays an important role in interactions of both dendritic cells 18 and NK cells 19. In NK cells, WASP co-localizes with F-actin at the activating immunologic synapse with target cells and undergoes phosphorylation 19,20. In WAS patients, F-actin accumulation at the activating immunologic synapses is less frequent, and both natural and antibody-mediated cytotoxicity are defective 19,20.

To assess the impact of the reversion, it is tempting to compare disease severity of the two brothers; however, this approach is not appropriate due to age-dependent variability of WAS.
Nonetheless, the disease of patient P1, consisting of thrombocytopenia and mild eczema without recurrent infection, is milder than expected for a (now) 11-year old patient with a truncating mutation.

Because previous reversions/corrections of WASP \((n = 8)\) were enriched in T cells, and barring an impediment to enrichment of revertant T cells in this patient, the restriction of his revertant gene and WASP-expressing cells to NK cells suggests that his reversion occurred in a cell or cells committed to the NK cell lineage. The rarity of reverse mutation events suggests that the WASP* NK cells are derived from one or a few original revertant cells. These findings have implications for gene therapy since they suggest that even the correction of a small number of stem cells with the capacity to differentiate into NK progenitors may suffice to substantially repopulate this lineage. Moreover, because NK cell protective capacities are germline-encoded with specialization determined late in differentiation \(^{21}\), the selected gene-corrected cells are anticipated to restore all functions of the compartment. Although correction of the NK cell defect would not be curative, a functioning NK compartment is expected to contribute to protection against viral infections and possibly malignancies, which are serious complications of the immunodeficiency in this disease. Thus, enrichment of gene-corrected cells in the NK compartment should be an integral target of gene therapy for WAS patients.
References


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