Nonrandom Inactivation of the X Chromosome in Early Lineage Hematopoietic Cells in Carriers of Wiskott-Aldrich Syndrome

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The Wiskott-Aldrich syndrome (WAS) is an X-linked (Xp11.22) recessive immunodeficiency syndrome characterized by susceptibility to opportunistic and pyogenic infections, thrombocytopenia, and eczema. Previous studies of obligate carriers of WAS documented that nonrandom inactivation of the X chromosome carrying the defective gene is observed in all peripheral blood cells. The existence of both abnormal platelets and lymphocytes is consistent with a defect that affects early hematopoietic precursors. We isolated CD34+ hematopoietic progenitor cells collected from obligate carriers of WAS by apheresis and used polymerase chain reaction analysis of a polymorphic variable number of repeats (VNTR) within the X-linked androgen receptor to document nonrandom inactivation. These data show that nonrandom inactivation of the X-chromosome in WAS-obligate carriers occurs early during hematopoietic differentiation.

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MATERIALS AND METHODS

Human subjects. Peripheral blood leukocytes were obtained by apheresis from normal healthy females and two obligate carriers of the Wiskott-Aldrich Syndrome (WAS) with informed consent of the donors and with approval of the Institutional Review Board on human subjects. Both carriers had affected sons, brothers, and other male relatives with WAS.

Immunohemical reagents. Antisera and protein dye conjugates were from the following sources: biotin-labeled goat-antimurine IgG, phycoerythrin (PE)-labeled goat-antimurine IgG, and streptavidin-labeled PE were obtained from Southern Biotechnology (Birmingham, AL). Intravenous IgG was obtained from Immuno AG (Vienna, Austria).

Murine monoclonal antibody (MoAb) to CD34 was obtained unlabeled (HPCA-1) and labeled with PE (HPCA-2-P) from Becton Dickinson (San Jose, CA). The NC2 murine IgG1, antimurine MoAb was obtained from the Fifth International Leukocyte Workshop. Labeled MoAbs for fluorescence-activated cell sorting (FACS) analysis were obtained as follows: PE-labeled anti-CD20 (L27) and anti-CD5 (L17F12) and fluorescein isothiocyanate (FITC)-labeled anti-CD5 (L17F12), anti-CD8 (SK2), anti-CD7 (4H9), anti-CD25 (2AB), anti-CD57 (HNK-1), anti-CD38 (HB7), anti-CD10 (W8E7), anti-CD20 (L27), and anti-CD18 (L130) were from Becton Dickinson. FITC-labeled anti-CD45RA was obtained from Dakopatts (Copenhagen, Denmark).

Magnetic beads 450 Nm in diameter labeled with anti-CD19, anti-CD4, and anti-CD8 were obtained from Dynal, Inc (Great Neck, NY). The magnetic beads and kit components (MACS and MINI-MACS isolation set) used for isolation of CD34+ cells were obtained from Miltenyi Biotech GmbH (Bergisch-Gladbach, Germany).

Apheresis. The COBE Spectra Apheresis Unit with hardware for collection of white blood cells (COBE Corp, Lakewood, CO) was used to perform mononuclear cell apheresis. Briefly, about 7 to 10 L of blood were apheresed over 3 hours, and about 200 mL of a mononuclear cells concentrate with a hematocrit of 1% to 3% were collected. Mononuclear cells were greater than 80% according to automated differential performed in a Technicon Hematology Analyzer (Tarrytown, NY). Those with a hematocrit greater than 2% were further fractionated by centrifugation on a Ficoll gradient in the COBE 2991 for 30 minutes at 400g. The cells at the plasma-Ficoll interface were harvested and washed three times with 450 mL of normal saline during the automatic cycle. The final mononuclear cell fraction was used for analysis.
clear cell concentrate (MCC) was suspended in 50 mL of normal saline and contained 6 to 10 × 10^6 cells.

Cell Isolations

**Isolation of CD34+ cells.** With the exception of the HPCA-1 MoAb, all reagents used to fractionate the CD34+ cells from WAS carriers, normal females, and males were obtained from Miltenyi Biotec GmbH and used according to the directions supplied by the manufacturer. Two different anti-CD34 MoAbs were used. Clone HPCA-1 obtained from Becton Dickenson was used for one obligate carrier, and the controls isolated in parallel. Clone HPCA-2, 8G12, obtained from Miltenyi, was used for the second carrier. Briefly, the MCC obtained by apheresis and Ficoll-gradient centrifugation was divided into four T75 Flasks (Falcon Plastics, Caldwell, NJ) and incubated at 37°C for 1 hour to remove monocytes that adhered to the plastic surface. Nonadherent cells were combined, suspended in phosphate-buffered saline (PBS) containing 5 mM EDTA, and 0.5% (wt/vol) purified human IgG and incubated for 10 minutes at 37°C to saturate Fc receptors. The cells were then reacted with anti-CD34 MoAb (HPCA-1) for 15 minutes at 0°C and then with biotin-labeled goat-antimurine IgG, for 15 minutes at 0°C. This mixture was then incubated with streptavidin conjugated to magnetic beads (MACS) for 10 minutes at 0°C followed by incubation with streptavidin PE for 15 minutes at 0°C. The cells were then loaded onto a magnetic cell sorter column (MACS), the nonadherent cells eluted, and the magnetic field removed to recover the adherent cells. This fraction contained both CD34+ dim and CD34+ bright cells according to FACS analysis (Fig 1C). Thus, this fraction was further enriched for CD34+ positive cells by fractionation on the MINI-MACS column and elution with Hank's balanced salt solution (HBSS) containing 2% fetal calf serum (FCS; Gibco Life Technologies, Gaithersburg, MD) and 5 mM EDTA. The cells finally eluted consisted of greater than 92% CD34 bright cells according to FACS analysis; this cell fraction was used for both hematopoietic progenitor cell assays and X-chromosome inactivation assays. As a negative control, MCCs were labeled with an irrelevant murine IgG, anti-human MoAb (clone NC2). No CD34+ bright cells were obtained when cells were labeled and fractionated with this antibody in place of the CD34+ MoAb. 14

**Isolation of macrophages and neutrophils, CD19+ cells, CD4+ cells, and CD8+ cells.** This was accomplished by standard methods. Briefly, approximately 30 mL of venous blood was collected in acid citrate dextrose anticoagulant (ACD) and applied to a Ficoll gradient. After centrifugation at 400g for 11 minutes, the neutrophils were harvested as the most rapidly sedimenting fraction. Cells at the plasma-Ficoll interface were collected and washed three times with 50 mL of HBSS. The cells were resuspended in RPMI with 5% FCS and incubated in plastic flasks at 37°C for 30 minutes to remove monocytes that adhere to the plastic (Falcon T75 flasks). After removal of nonadherent cells, the adherent cells were incubated in 5 mL of 0.25% trypsin and 1 mL/mL EDTA (Gibco) for 5 minutes at 37°C, and the cells were removed with a sterile plastic scraper, suspended in RPMI containing 5% FCS, washed three times with RPMI containing 8% FCS, and DNA isolated from the cell pellet as described below. Nonadherent cells were combined, suspended in RPMI containing 5 mM/L EDTA and 0.5% (wt/vol) purified human IgG (Immuno AG), and incubated for 10 minutes at 37°C to saturate the Fc receptors. The cells were then reacted sequentially with anti-CD4, anti-CD8, and anti-CD19 MoAbs coupled to the 450 μm Dynal beads (Dynal). After each binding, the beads were washed and the cells were detached according to instructions provided by the manufacturer. The immunofluorescence fractionation CD4+, CD8+, and CD19+ cells exhibited 95% to 98% purity when analyzed by FACS with FITC-labeled anti-CD4, anti-CD8, and anti-CD19 MoAbs, respectively (data not shown). No additional characterization of the neutrophil or monocyte fraction was done. These cell populations were examined for random versus nonrandom X inactivation (see below).

**FACS analysis.** The CD34+ cells were analyzed for purity by FACS analysis by staining 10^7 cells with 20 μL of the anti-CD34 MoAb HPCA-2 (clone 8G12, IgG1 specific) for 15 minutes at 0°C, washing two times with FACS buffer (Becton Dickinson) (PBS containing 2% bovine serum albumin [BSA], 3% FCS and 0.02% NaN3), and then staining with 20 μL of PE-labeled goat-antimurine IgG. The cells were then washed again twice with ice-cold PBS, fixed in 2% paraformaldehyde (Sigma, St Louis, MO) and analyzed by FACS within 24 hours with the acquisition gate for the FACS analysis set with unfixed unstained cells to exclude cell aggregates and debris.

Staining with other antibodies for FACS analysis to determine phenotypes was done as follows: about 10^5 CD34+ cells fractionated with HPCA-2 and containing about 80% CD34+ and the remainder CD34- were labeled with HPCA-2 as described above. Mouse serum was added to a final concentration of 20% murine serum (Gibco) for 15 minutes and the cells washed with PBS. They were then incubated in “V” microtiter wells for 15 minutes at 0°C with 20 μL of PE-labeled goat-antimurine IgG, washed twice with FACS buffer (Becton Dickinson) (PBS containing 2% BSA, 3% FCS, and 0.02% NaN3). The FITC-labeled MoAbs were then added at concentrations indicated by the manufacturer and incubated for 15 minutes at 0°C. The cells were then washed again twice with ice-cold PBS, fixed in 2% paraformaldehyde, and analyzed by FACS within 24 hours. The gates for the FACS analysis were set with unfixed unstained cells as described above.

**Hematopoietic progenitor cell assay.** Burst-forming unit-erythroid (BFU-E), colony-forming unit granulocyte-macrophage (CFU-GM) and colony-forming unit granulocyte-erythroid-monocyte-megakaryocyte (CFU-GEMM) progenitor cells were quantified as previously described. 17 Cells were mixed with methylcellulose assay media containing 30% FCS, 3 U/mL erythropoietin, 5% serum containing PHA-LCM, 1% BSA, 0.9% methylcellulose, 10 μM 2-mercaptoethanol, and 2 mM glutamine (Stem Cell Technologies, Vancouver, BC, Canada) and plated onto 35-mm plastic dishes (Nunc Inc, Naperville, IL). The mononuclear cells were plated at a concentration of 5 × 10^5 cells/plate. The CD34+ cells were plated at concentrations of 5 × 10^4 to 1 × 10^5 cells/plate. The cells were incubated at 37°C in air supplemented with 5% CO2 and saturated with humidity. BFU-E, CFU-GM, and CFU-GEMM were enumerated by standard criteria at 14 to 15 days. 15

**Isolation of DNA.** Genomic DNA was isolated from blood and cells as follows: between 1 × 10^10 to 1 × 10^11 cells were digested 2 to 4 hours at 37°C in 467 mL of 10 mM TRIS-10 mM EDTA-10 mM NaCl, pH 7.4, containing 15 μL of proteinase K (10 mg/mL) and 50 μL 20% sodium dodecyl sulfate (SDS). The DNA was then extracted with phenol/chloroform by standard methods, concentrated, and washed three times with TE buffer on a Centricon 100. Alternatively, DNA was precipitated with two volumes of absolute ethanol and 1/10 volume of 3 M sodium acetate, centrifuged, and resuspended in H2O. DNA was quantified by spectrometry at 260 and 280 nm.

DNA was extracted from buccal swabs of oral epithelium as follows: sterile rubber policemen were rubbed on the inside of the mouth and put into 5 mL of HBSS. Cells were collected by centrifugation, and the pellet suspended in 467 μL of 10 mM TRIS-10 mM EDTA-10 mM NaCl, pH 7.4 containing 15 μL of proteinase K (10 mg/mL), and 50 μL 20% SDS. The reactions were incubated overnight 2 to 4 hours at 37°C. The DNA was extracted and quantified as above.

**X-chromosome inactivation assays of normal and WAS carriers.** Briefly, PCR amplification was done on genomic DNA digested with

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and without Hpa II with primers that bracket a polymorphic VNTR marker located in the second exon of the human androgen receptor gene as described by Allen et al. This was done as follows: A reaction containing 200 ng of DNA and 6U of Taq I was digested for 1 hour at 37°C. The reaction mixture was divided in half and one aliquot further digested with 8U of Hpa II for 1 hour at 37°C. Both reactions were then amplified with primers that have the following sequence: A. 5'-GCT GTG AAG GIZT GCT 3' B. 5'-TCC AGA ATC TGT TCC AGA GCG TGC-3'. Amplification was done in an 80 μL volume and contained 10 mM TRIS HCl, 50 mM KCl, 0.01% gelatin (wt/vol), 2.5 mM MgCl2, 1 U Taq (AmpliTaq, Perkin Elmer, Norwalk, CT), deoxy nucleotide triphosphate at a final concentration of 250 μM, 3 × 10-3 μCi of 32P deoxycytidine triphosphate (dCTP) and 10 μM primers. Amplification conditions included a "hot start" at 80°C; specifically, primer B was added, the temperature raised to 80°C and then primer A added. DNA was denatured at 95°C for 5 minutes and amplified for 28 cycles (95°C; 45 seconds, 60°C; 30 seconds, 72°C; 30 seconds, extend 5 minutes 72°C and store at 4°C). A Perkin Elmer 480 Thermocycler (Perkin Elmer, Norwalk, CT) was used for all amplifications.

For analysis, 2 μL of the PCR product was mixed with 6 μL of DNA sequencing stop buffer (Sequenase Kit, Stratagene, Cambridge, MA), heated for 5 minutes at 90°C and placed on ice. Each mixture was then electrophoresed on a denaturing 8% polyacrylamide with 4 M urea run for 5 hours at 50 W. The gel was transferred to 3M Whatman filter paper and the bands detected following exposure to AR-X ray Film (Fuji, Sigma) overnight at -70°C.

RESULTS

Isolation of CD34+ Cells From Normal Individual and WAS Carriers

FACS analysis with anti-CD34 MoAb of the various cell fractions obtained during the enrichment process is shown in Fig 1. Figure 1A is the FACS analysis of MCC fraction after apheresis, Fig 1B is the FACS analysis after removal of macrophages and the first MACS fraction, and Fig 1C is the FACS analysis of cells after one separation by MACS and one by MINI MACS. This fraction contained both CD34+dim and CD34+bright cells. This observation is probably caused by heterogeneity in this cell fraction because Civin17 has previously noted that the CD34 antigen is on maturing hematopoietic progenitor cells. After a third fractionation on magnetic beads (MINI-MACS) (Fig 1D), the majority of the cells were CD34+bright and the yield was about 1.5 × 106 cells that were 92% CD34+ for both WAS carriers and normal controls. Others have reported a similar yield of CD34+ cells obtained by immunoaffinity enrichment of peripheral blood leukocytes obtained by leukopheresis.18

Phenotyping of CD34+ Cells Isolated From Normal Individuals

FACS analysis was performed. The fraction containing CD34+bright and CD34+dim cells (Fig 1C) were isolated as described in Materials and Methods from two normal individuals. For these analyses, the FITC-labeled MoAbs specific for various cell surface markers were used after staining of the CD34+ cells. This cell fraction contained cells staining for markers found on committed cells (22% CD5, 23% CD7, 45% CD8, 45% CD20, 22% CD25, 3% CD57, 48% CD10, and 52% CD18). More important, the cell fraction stained for markers of uncommitted cells (86% CD38 and 80% CD45RA). The results indicated that the fraction of CD34+ cells isolated at an intermediate step were enriched for cell surface markers found on cells uncommitted in terms of differentiation towards cells that have defined functional and morphologic characteristics.

Hematopoietic Progenitor Cell Assays on CD34+ Cells

To document enrichment of hematopoietic progenitor cells in the fraction of the CD34+ cells, various cell fractions were analyzed for BFU-E, CFU-GM, and CFU-GEMM. Re-
cells derived from progenitors with either X will be represented and a doublet pattern will be observed. However, if a nonrandom selection has occurred, a singlet pattern will be observed. Although it is possible that cells from any single lineage may show some skewing by chance (a corollary of the Lyon hypothesis), it is unlikely that a uniform pattern of X-inactivation would be observed within cells of different lineages, and hence, observation of such a pattern is consistent with the conclusion of nonrandom section. 19

The random (doublet) and nonrandom (singlet) X-chromosome pattern is evident in both a normal (Fig 3A) and an obligate WAS carrier (Fig 3B) in DNA extracted from whole blood as well as neutrophils, monocytes, and CD4+, CD8+, and CD19+ cells (lanes 1 through 6). These results confirm the observation of nonrandom inactivation of cells of different hematopoietic lineages as well as DNA derived from whole blood. Analysis of obligate carriers as well unaffected individuals within the extended family showed segregation of restriction fragment-length polymorphism (RFLP) alleles linked to the WAS gene consistent with the distribution of WAS within the pedigree. 3

As a control, DNA extracted from the blood of a normal male was analyzed identically and found to exhibit the expected pattern, namely a singlet that is consistent with one active X-chromosome (Fig 3B, lane 7). These results also confirm that in WAS all hematopoietic lineage are affected by the WAS mutation. The identical PCR analysis

Fig 3. X-chromosome inactivation pattern in a normal female and a WAS carrier. Autoradiographs of PCR products from normal female after amplification with 32P dCTP and electrophoresis of the PCR products on denaturing polyacrylamide gel electrophoresis (see Materials and Methods). (A) Normal female; (B) WAS carrier. DNA isolated from whole blood (lane 1), neutrophils (lane 2), macrophages (lane 3), CD4+ cells (lane 4), CD8+ cells (lane 5), and CD19+ cells (lane 6) were analyzed. DNA restricted with Rsa I and Hpa II is indicated by a (+). In A, lane 7 is whole blood from a WAS carrier, lane 8 contains no DNA; in B, lanes 7 and 8 are DNA extracted from whole blood of a normal male and a normal female, respectively. Lane 9 contains no DNA. DNA restricted with Rsa I only is indicated by a (+). The isolation of the cells was performed by Ficoll-gradient centrifugation, and immunofluorescence fractionation of cells and electrophoresis were done as described in Materials and Methods.
has been conducted on four other documented WAS pedigrees and in all cases, the same pattern of random and nonrandom inactivation was established for neutrophils, macrophages, and CD19⁺, CD4⁺, and CD8⁺ cells isolated as described in Materials and Methods from obligate WAS carrier and noncarrier family members. Finally, in dilution experiments with CD4⁺, CD8⁺, and CD19⁺ cells isolated from normal females, it has been possible to detect the random pattern of X-chromosome inactivation with the DNA extracted from as few as 1,000 cells (data not shown).

The same experiment was performed on CD34⁺ cells isolated from a WAS carrier and two controls, a normal female and a normal male. These results are presented in Fig 4, A and B. DNA isolated from whole blood and CD34⁺ cells of a normal female and male (Fig 4A, lanes 1 and 2) and CD34⁺ cells (Fig 4A lanes 3 and 4) exhibit the expected patterns of X-chromosome inactivation. The WAS carrier in contrast exhibited a pattern of nonrandom inactivation in DNA extracted from blood and CD34⁺ cells (Fig 4A, lanes 5 and 6), but not in DNA extracted from buccal swabs of the oral mucosa (Fig 4A, lane 7). Identical results were obtained in a second obligate WAS carrier except the upper bands rather than the lower bands are missing (Fig 4B). Prolonged exposure failed to show any bands where the lower ones should have appeared, but the DNA extracted from the buccal swabs always exhibited a pattern consistent with random inactivation. This observation is consistent with the corollary to the Lyon hypothesis, which states that when nonrandom inactivation occurs in a critical tissue of a female, only cells derived from the cell lacking the mutant gene survive.

DISCUSSION

The phenotype of the Wiskott-Aldrich syndrome is variable among affected families and has even manifested itself as thrombocytopenia without other stigmata of the syndrome. In all affected families, where mapping is feasible, the WAS gene defect has been found to localize to an area between DXS255 (Xp11.22) and TIMP (Xp11.3). Recently, a close linkage was shown between WAS and OATL1 (311240) loci (maximum logarithm of the odds ratio, 6.08; at theta, 0). This finding localized TIMP, OATL1, and the WAS loci proximal to TIMP. DNA has been examined by RFLP, and in ~80 WAS-affected kindreds, the defective gene has recently been identified. As platelets and T cells are the most prominently affected blood cells in WAS, it has been difficult to put forth a unifying hypothesis to explain all the observations that have been made on the blood of affected males. It appears that a cytoskeletal abnormality underlies the bizarre morphology of the T cells and platelets in this disease. One consistent immunologic abnormality has been observed in affected males and that is the inability of their T cells to proliferate properly when stimulated by immobilized anti-CD3. The response to anti-CD3 requires the bundling of cytoskeletal actin in contrast with other mitogenic stimuli to T cells.

In this study, we used a clonality assay based on X-chromosome inactivation to characterize whether in known female WAS carriers, blood cells exhibiting markers of early lineage commitment also carried the WAS mutation. Specifically, we used a PCR-based assay that takes advantage of a highly polymorphic (greater than 90%) trinucleotide repeat that is in the coding region of the first exon of the human androgen receptor gene and correlates with X-chromosome inactivation. The trinucleotide repeat is closely linked to four methylation sites that meet the obligate requirements for an X-chromosome inactivation assay that is based on variably methylated sites. The principles of the assay are as follows: (1) during embryogenesis in females, there is random inactivation of one X chromosome in each cell, and the pattern of X-chromosome inactivation is maintained in all progeny of that cell; and (2) inactivation of the X chromosome is accompanied by changes in methylation patterns that can be delineated with methylation sensitive endonuclease restriction enzymes such as Hpa II. In the assay used in the present studies, the active X chromosome is unmethylated and the inactivated X chromosome is methylated at CpG-rich regions. Two of the four methylation sites are detected by digesting the DNA with Hpa II before amplifying with primers that flank the methylation site. If
digestion occurs, there is no PCR amplification of the repeat sequence. 17

With this assay we were able to show a pattern of nonrandom inactivation in all the blood cell lineages in female carriers of WAS as opposed to normal females (Fig 3). Because of the sensitivity of the PCR technique, the pattern of random and nonrandom inactivation can be detected in as few as 103 cells. This assay was used to determine whether the same pattern of nonrandom inactivation would be observed in a population containing stem cells. These cells were obtained by enrichment with a MoAb that detects CD34+ cells (Fig 1). As a control, we used buccal mucosal cells as a source of somatic tissue to confirm that the pattern observed in the DNA isolated from nonhematopoietic cells of the WAS carrier was consistent with nonrandom inactivation. In DNA isolated from buccal mucosal epithelial cells from females shown not to be WAS carriers (n = 10) and in normal females (n = 10), a pattern consistent with random inactivation of the X-chromosome similar to what is presented in Figure 4A, lane 7, was found (data not shown). Skewing of the pattern, as noted by others, is consistent with a conclusion that even in normal females, random inactivation is not always balanced. As the present assay is qualitative rather than quantitative, it was not possible to estimate the range of deviation from random inactivation in DNA extracted from mucosal cells. In contrast, we found that when hair roots were used as a source of somatic tissue in the same normal females and the females within WAS pedigrees determined to be noncarriers, we observed the patterns consistent with both random and nonrandom inactivation, with no correlation to carrier status within WAS kindreds. 18 The latter observation has also been observed when peripheral blood cells of normal females have been used as a source of genomic DNA for analysis of random X-chromosome inactivation.9,17,19,20

Because blood cells, other than T lymphocytes and platelets, do not manifest abnormal phenotype or function in affected WAS males, it was surprising to find that all blood cell lineages exhibit nonrandom X-chromosome inactivation in obligate female heterozygous carriers of the WAS mutation. This finding raised the question as to whether hematopoietic progenitors are affected by the mutation, and the results of the present study indicate that such is the case. Although the population of CD34+ cells isolated for the purposes of this study from peripheral blood cells exhibit markers of early lineage commitment, if even as few as 1% of these cells were uncommitted progenitor cells, there would have been a sufficient number to show random X-chromosome inactivation. No random X-chromosome inactivation was found in the CD34+ cells of those female members of WAS kindreds who were obligate heterozygous WAS carriers. Although the present X-chromosome inactivation studies have provided insights as to when the WAS mutation is expressed in hematopoietic cell lines in differentiation, an explanation for the survival advantage of those stem cells not bearing the X-chromosome with the WAS mutation awaits identification of the role of the WAS gene.

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X-CHROMOSOME INACTIVATION IN CARRIERS OF WAS

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