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Leukemia associated mutant Wilms' tumor gene 1 protein promotes expansion of human hematopoietic progenitor cells

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ABSTRACT

The transcription factor Wilms' tumor gene 1 (WT1) is highly expressed in the majority of leukemias, suggesting a role in leukemogenesis. Acquired WT1 mutations are reported as an independent predictor of poor clinical outcome, and mutations resulting in deletion of the entire DNA-binding zinc-finger domain (WT1delZ), is the most common type. The aim of this study was to study cellular effects of WT1(delZ) that may contribute to an oncogenic phenotype. We found that expression of WT1(delZ) supported proliferation of human hematopoietic $CD34^+$ progenitor cells. Moreover, WT1(delZ) transduced cells expressed erythroid markers, including raised levels of STAT5, independently of addition of erythropoietin. At the global gene expression level, WT1(delZ) caused upregulation of genes related to cell division and genes associated with erythroid maturation, in the absence of added erythropoietin. Our results indicate that WT1(delZ) promotes cell proliferation and expansion of progenitor cells, consistent with a possible role in leukemogenesis.

1. Introduction

The WT1 gene encodes a transcription factor with four DNA-binding carboxyl terminal zinc-fingers, whereas the amino terminal part contains domains that mediate self-association, RNA recognition, and transcriptional regulation [1]. In adult human hematopoiesis, WT1 is expressed in a small subset of progenitor cells, but is undetectable in mature blood cells, consistent with a role for WT1 in hematopoiesis [2–4]. In mice, WT1 was reported as not absolutely required for murine hematopoiesis [5], but in competitive reconstitution assays, embryonic stem cells without WT1 failed to contribute to the hematopoietic system, indicating an advantage for WT1-expressing cells, as compared to WT1-null cells [6]. Recently, the role for WT1 in adult murine hematopoiesis was investigated by inducible deletion of the WT1 gene in vivo, leading to rapid development of aberrant hematopoiesis with complete failure of erythrocyte formation, due to intrinsic defects of erythroid progenitors [7].

Although initially characterized as a tumor suppressor in Wilms' tumor, aberrantly high expression of WT1 is detected in several forms of cancer, including breast cancer and some cases of Wilms' tumor itself [1,8], paradoxically suggesting an oncogenic role for WT1. WT1 is also highly expressed in the majority of lymphoid and myeloid leukemias [9], although the mechanism underlying this effect remains unexplained.

Acquired somatic WT1 mutations in leukemia were first reported more than 15 years ago [10–12]. In recent years, several larger studies have confirmed these early data. Thus, WT1 mutations are present in approximately 10–15% of cytogenetically normal (CN) adult AML cases at diagnosis, and in most studies the presence of WT1 mutations was reported as an independent predictor for poor clinical outcome [13–18], and reviewed in [19,20]. The overrepresentation of WT1 mutations in the subgroup of CN AMLs may suggest a driver role for the mutations in leukemogenesis. Similar types of WT1 mutations have also been found in pediatric AML [21–23].

The WT1 mutations are heterozygous frameshift mutations, or substitutions, predominantly in exon 7 and 9, respectively, predicted to encode WT1 proteins with impaired DNA-binding ability. Frameshift mutations in exon 7 are the most common class, encoding truncated proteins lacking the DNA-binding zinc-finger domain. We and others have previously shown that WT1 with deleted zinc-fingers, WT1(delZ), may have effects on the cellular phenotype in spite of no direct regulation of target genes, due to loss of DNA-binding capacity [24,25].

Herein, we extend our previous investigations by the use of prolonged suspension cultures and replating clonogenic assays, with and without exogenous delivery of erythropoietin. We show that

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expression of WT1(delZ) in primary CD34+ hematopoietic progenitors results in enhanced proliferation, both in the presence and absence of added erythropoietin. Moreover, we demonstrate that WT1(delZ), in the absence of added erythropoietin, entails expression of erythroid and proliferative markers, including activation of STAT5.

2. Materials and methods

2.1. CD34+ progenitor cells and retroviral transduction

Human CD34+ progenitor cells were extracted from umbilical cord blood as previously described [26].

The MSCV-based retroviral vector MIG contains an internal ribosomal entry site (IRES) and enhanced green fluorescence protein (eGFP). MIG-WT1(+/−) and MIG-WT1(delZ) contain the human cDNA for WT1(+17AA/−KTS) and WT1(delZ) (+17AA isoform with deleted zinc fingers, thus encoding the N-terminal part, amino acids 1–326) as described previously [24]. Empty MIG vector was used as control. The transduction efficiencies were 40% (control), 20% (WT1) and 38% (WT1(delZ)) respectively (mean values).

2.2. Suspension cultures

The FACS sorted cells were seeded at 400,000 cells/ml in StemSpanSFEM with added StemSpan CC100 (containing Flt3-ligand, Stem Cell Factor, IL-3, and IL-6), 100 ng/ml thrombopoietin (hTPO) and 20% fetal bovine serum, all obtained from Stemcell Technologies. Cells were split into two cultures with and without 3 U/ml erythropoietin (Eprex, Janssen-Cilag, Sollentuna, Sweden) as additive. The cell cultures were carefully monitored and fresh complete medium with or without erythropoietin was added when needed to redilute the cells to a concentration of 400,000 cells/ml.

2.3. Human colony-forming cell assay

GFP+ sorted cells were plated to 35 mm dishes (500 cells/ml), and cultured in triplicates in methylcellulose MethoCult® H4434 Classic (containing 3 U erythropoietin/ml) or MethoCult® GF+ H4535 (without erythropoietin) (Stemcell Technologies, Vancouver, Canada). Erythroid colonies, identified as hemoglobinised cells with growth characteristics of BFU-E or CFU-E, were determined as CFU-Ery. CFU-Myelo includes CFU-GM, CFU-M and CFU-G. Following scoring after 10 days, cells were resuspended in IMDM, after which total cell number and cell viability were determined by counting in Bürker chambers with trypan blue exclusion. All cells in the methylcellulose dishes were replated in new methylcellulose mixture with the same composition as previously, for determination of replating colony-forming capacity.

2.4. Real-time quantitative PCR

Real-time quantitative PCR (qPCR) analysis was performed using a StepOnePlus™ Real-Time PCR (Applied Biosystems, Foster City, CA, USA) and standard protocols. Probes for erythropoietin receptor (EpoR) (Hs00181092_m1), Epo (Hs01071097_m1), STAT5A (Hs00559643_m1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1) were all purchased as TaqMan® Gene Expression Assays (Applied Biosystems). Relative quantitation was calculated based on the $\Delta\Delta C_T$ method [27] using GAPDH as calibrator. Parallelism of standard curves of the test and control samples was confirmed.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Cells grown in suspension cultures for 14 days were lysed after which the levels of adult hemoglobin (HbA) and fetal hemoglobin (HbF) in cell samples were quantified by ELISA as described in [28].

2.6. Western blot

A polyclonal rabbit anti-WT1 antibody, WT1(180) (sc-846), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (6C5) (sc-32233), and anti-actin (C-2) (sc-8432) were purchased from Santa Cruz Biotechnology, CA, USA. Anti-STAT5A (#06-553), anti-STAT5B (#06-554) and anti-phospho-STAT5A/B (Tyr694/699) (#05-495) were purchased from Upstate Biotechnology, Lake Placid, NY, USA. HRP-conjugated secondary antibodies, goat-anti-rabbit IgG (H+L) and goat-anti-mouse IgG (H+L), were purchased from BioRad, Hercules, CA, USA. For quantification of band intensities, a Molecular Imager ChemiDoc XRS+ with Image Lab Software (BioRad, Hercules, CA, USA) was used.

2.7. Illumina array and gene set enrichment analysis

RNA from four independent experiments was subjected to whole-genome gene expression analysis using the Illumina platform HumanHT-12 v4 expression BeadChip Kit (Swegene Center for Integrative Biology, Lund University). Statistical analysis of differences in gene expression between WT1(delZ) expressing cells and control cells was made using the Significance Analysis of Microarrays (SAM) [29]. Illumina data are available in the GEOarchive (http://www.ncbi.nlm.nih.gov/geo) GEO accession number GSE47560. A heatmap showing 100 probe-sets with the highest differential gene expression (control vs WT1(delZ) cells) was made.

To perform gene set enrichment analysis, we used the program RenderCat [30] (based on the Zhang C goodness-of-fit test). To identify marker genes for different types of blood cells, we used the d-map compendium [31], a comprehensive microarray data set containing gene expression profiles of various types of sorted blood cells from healthy individuals (Affymetrix U133A arrays). Marker genes were identified by comparing each cell type with all other cell types using Smyth's moderated *t*-test [32]. To match genes across the Illumina and Affymetrix microarray platforms, we used Entrez Gene IDs provided in the manufacturers' annotation files. Gene sets used in the enrichment analysis are provided as Online Supplementary Material.

2.8. Transient transfection

293T/17 cells (ATCC #CRL-11268) were transiently transfected with STAT5A plasmid (GeneCopoeia, MD, USA) and co-transfected with increasing amounts of pcDNA3/WT1 or pcDNA3/WT1(delZ). Equivalent amount of DNA was used for all transfections with input of empty pcDNA3-vector as equivalent weight, when needed. After 48 h the cells were harvested, lysed and used for Western blot analysis. For quantification of band intensities, a Molecular Imager ChemiDoc XRS+ with Image Lab Software was used.

2.9. Statistical analysis

Analysis of the proliferation rate for the CD34+ cells in suspension cultures (Fig. 1) was performed by the use of a linear mixed model of log 10-transformed numbers with random effects for repeated measurements. This model showed a significant interaction between cell cultures (control/WT1/WT1(delZ) and time (Fobs = 2.61, ndf = 16, ddf = 66, $p < 0.0033$). Post hoc analyses using the joint normal distribution of the estimates ("single-step" in multcomp) with all three pairwise comparisons at each time point was calculated and all statistical significant differences were indicated in Fig. 1A and C. The effects on clonogenic growth after plating and replating in methylcellulose were analyzed by the use of a generalized linear mixed model for the binomial response. This model showed that the log-odds differ between groups (likelihood ratio-test, $p < 0.001$). Significant post hoc pairwise comparisons between log-odds for control and WT1 and control and WT1(delZ) are indicated in Fig. 2A–D. The effects on the amount of hemoglobin A was analyzed with a quasipoisson-model with log-link and log(total protein) as offset. This showed differences between groups in the amount of HbA/amount of total protein (Fobs = 14.3, ndf = 2, ddf = 5, $p = 0.0085$). Statistical significant differences obtained by post hoc comparisons are indicated in Fig. 4A and B. Mixed model analyses were done using R ver 3.0.0 [33] with lme4(0.999999-0) [34]. Post hoc analyses were done using multcomp (multcomp_1.2-17) [35].

3. Results

3.1. Expression of WT1(delZ) results in increased proliferation in liquid culture

Previous data indicate that WT1(delZ) has distinct effects on myeloid and erythroid growth upon expression in progenitor cells, compared to wild-type WT1 [24]. To further investigate the effects of WT1(delZ) on expansion and proliferation, transduced cells were cultured in suspension supported by early- and late-acting cytokines (Section 2) to achieve a rapid and strong proliferative response of the progenitors. As a comparison to WT1(delZ) we chose full length–KTS WT1 isoform (WT1+/−) since the −KTS isoform is reported to show the most prominent DNA-binding ability. To evaluate the dependence on exogenous erythropoietin for proliferation, cultures were grown with or without added erythropoietin. With no addition of erythropoietin, control cells showed a 14-fold and 900-fold expansion after 7 and 21 days, respectively (Fig. 1A). The proliferation of WT1-transduced cells showed a reduced expansion rate, as compared to control cells. Cells expressing WT1(delZ), however, showed an enhanced proliferation during the latter part of the culture period: on day 21, WT1(delZ) cells showed a 4600 fold expansion (as compared to