The effects of Gremlin1 on human umbilical cord blood hematopoietic progenitors ☆

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ABSTRACT

Bone morphogenetic proteins (BMPs) support malignant hematopoiesis in CML. Conversely, the multi-functional BMP antagonist Gremlin1 supports self-renewing cancer stem cells of other malignancies. Inhibition of BMP signaling in CML, or of Gremlin1 in solid tumors, may therefore have therapeutic potential. However, since BMPs regulate hematopoietic stem cell (HSC) decisions in the stem cell niche, it is necessary to determine how Gremlin1 influences normal HSC. We examined the effects of Gremlin1 on long-term culture-initiating cells (LTC-IC) and transplantable hematopoietic stem cells (SCID-repopulating cells: SRC) in human umbilical cord blood. Gremlin1 inhibited BMP signaling, downregulated BMP-6 and cyclin E2 expression and upregulated hairy and enhancer of split-1 (HES-1; a Notch transcriptional target) and Hedgehog interacting protein-1 (HHIP-1; an inhibitor of Hedgehog signaling). The functional effects of Gremlin1 on SRC, i.e. skewing of their myelopoietic: lymphopoietic potential towards B lymphopoiesis without affecting long-term engraftment potential, were entirely consistent with changes in gene expression induced by Gremlin1. Since both BMPs and Gremlin1 are secreted by osteoblasts in vivo, our studies provide potential insights into the molecular regulation of hematopoiesis in the stem cell niche. These results also suggest that Gremlin1 (and possibly its mimetics that may be developed for therapeutic use) may not adversely affect normal human hematopoietic stem cell survival, though they may reduce their myelopoietic potential.

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Introduction

Gremlins are multi-functional, secreted, cysteine-knot proteins which antagonize bone morphogenetic protein (BMP) -2, -4 and -7 by direct binding. Additionally, Gremlins have diverse effects on several pathways including Sonic hedgehog (SHH), fibroblast growth factor (FGF), Wnt and Notch ligand expression and signaling, cell cycle control proteins and cytokine signaling [1–3]. Gremlin1 is expressed in normal hematopoietic tissues including the spleen [4], and has BMP-independent effects on endothelial cells and monocytes [5,6]. A wide spectrum of human malignancies, including diffuse large B-cell lymphoma, express Gremlin1 [7].

Recent studies showed that increased BMP-2 and -7 expression in bone marrow stroma, together with increased expression of their receptor BMPR1b on CD34+ cells, contributes to the survival and proliferation of malignant progenitors in chronic myeloid leukemia (CML) [8]. Surprisingly and in contrast, Gremlin1 itself promotes other malignancies by inhibiting cancer stem cell differentiation via antagonism of BMPs as well as inhibition of the cell cycle component p21 [9,10], and stimulating angiogenesis [11]. These observations suggest the therapeutic potential of Gremlin1 inhibitors in solid tumors, and conversely, BMP inhibitors in CML and other malignancies.

Importantly, BMPs also influence development, survival and cell fate decisions of normal hematopoeitic stem cells (HSCs) [12–20]. Since Gremlin1 is produced by and regulates BMP-mediated development and differentiation of osteoblasts [21,22], cells that influence HSC niche function [23–28], it is critical to determine the molecular and functional effects of Gremlin1 on normal human HSC, for development of therapeutic approaches based on the Gremlin1/BMP pathway. We therefore examined the molecular and functional effects of Gremlin1 on primitive human myelopoietic progenitors (long-term

culture-initiating cells: LTC-IC) and transplantable hematopoietic stem cells (SCID-repopulating cells: SRC).

Methods

These studies were approved by the institutional Human Subjects Committee (IRB) and Institutional Animal Care and Use Committee (IACUC).

Sources of materials, cells and animals

Umbilical cord blood (UCB) was obtained from the National Disease Research Interchange (NDRI; Philadelphia, PA) and from the Red Cross National Cord Blood Program, Portland, OR. Ficoll-Hypaque (specific density 1.077) was from Sigma Aldrich, St. Louis, MO. MiniMACS kit and columns were from Miltenyi Biotec, Auburn, CA. PE-conjugated antibodies against CD2, CD7, CD10, CD19, CD33 and CD38 were from BD Biosciences Pharmingen, San Diego, CA. Recombinant human thrombopoietin, Flt-3 ligand, stem cell factor, macrophage inflammatory protein-1α, 1 ng/mL interleukin (IL)-6, IL-8, monocyte chemotactic protein-1, vascular endothelial growth factor, BMP-4, BMP 2/7 heterodimer and Gremlin1 were all from R&D Systems, Minneapolis, MN. Granulocyte colony-stimulating factor was from Amgen, Thousand Oaks, CA. N-desulfated, N-reacetylated heparin (NDSNAc, which retains O-sulfation) was from Associates of Cape Cod, Inc., East Falmouth, MA. Microporous collagen-coated 0.4-μm Transwell® inserts were from Corning Inc., Corning, NY. NOD-scid-IL2Rg^{null} (NSG) mice were obtained from The Jackson Laboratory, Bar Harbor, ME, and housed in a restricted barrier facility at the Minneapolis VA Medical Center. All labeled antibodies used for flow cytometric analysis of bone marrow from transplanted mice were obtained from BD Pharmingen. PicoPure RNA Isolation Kit was from Arcturus, Mountain View, CA, Single Primer Isothermal Amplification (SPIA) Ovation Pico WTA System from NuGen, San Carlos, CA, Stem Cell Signaling RT^2 Profiler PCR array and QuantiTect SYBR® Green PCR kit from Qiagen, Valencia, CA, and the ABI 7900HT Sequence Detection System from Applied Biosystems, Forest City, CA.

Umbilical cord blood processing and cell enrichment

Mononuclear cells were separated by Ficoll-Hypaque centrifugation. CD34+ cells were obtained using the MiniMACS CD34 Multisort kit and a MACS™ LS column, followed by a second enrichment (MS column). CD34+/CD38− cells were obtained using CD38-PE antibody and anti-PE magnetic microbeads, followed by negative selection (MS column). CD34+/CD38−/lin− cells were obtained using PE-conjugated antibodies against CD2, CD7, CD10, CD19, CD33 and CD38 and anti-PE magnetic microbeads, followed by negative selection (MS column).

Culture medium

Medium consisted of long-term bone marrow culture medium [29] supplemented with 10 ng/mL each of recombinant human thrombopoietin, Flt-3 ligand, monocyte chemotactic protein-1 and vascular endothelial growth factor; 200 pg/mL each of stem cell factor and macrophage inflammatory protein-1α, 1 ng/mL interleukin-6, 5 ng/mL interleukin-8, 250 pg/mL granulocyte colony-stimulating factor and 5 μg/mL N-desulfated, N-reacetylated heparin (referred to as OS-HS, which we have previously shown to support long-term in vitro maintenance of LTC-IC [29–31] and short-term maintenance of serially transplantable SRC [32]).

Quantitative real time PCR (qPCR)

CD34+/CD38−/lin− cord blood cells were cultured in medium alone or medium supplemented with 50 ng/ml Gremlin1 for 24 h. Total cellular RNA was isolated from the cells with the PicoPure RNA Isolation Kit. RNA quality and concentration were assessed on an Agilent RNA Pico chip using an Agilent 2100 bioanalyzer. RNA (10 ng per sample) was linearly amplified and converted to cDNA using a Single Primer Isothermal Amplification (SPIA) Ovation Pico WTA System. The quality and size of cDNA were examined using an Agilent RNA Nano chip, and quantified by spectrophotometry.

Initially, target genes were identified using the Stem Cell Signaling RT^2 Profiler PCR array, using RNA samples from CD34+/CD38−/lin− cells isolated from 4 umbilical cords and cultured in medium alone or medium + Gremlin1. Individual genes were further analyzed by qPCR, in addition to other genes suggested to be involved in Gremlin1 signaling.

qPCR was performed on 25 ng SPIA cDNA using QuantiTect SYBR® Green PCR kit on an ABI 7900HT Sequence Detection System. PCR conditions were 95° for 15 min followed by 45 cycles of 95° (15 s) and 60° (40 s) using 375 nM gene specific primers. Primers were designed on the Primer3 website (http://bioinfo.ut.ee/primer3-0.4.0/primer3/input.htm) using parameters specified by us previously [33]. Primer sequences are listed in Supplemental Table 1. Comparative expression of target genes was assessed using the $2-\Delta\Delta CT$ method by comparing their expression to β-actin [34]. Values that were extreme outliers, often because of inconsistent amplification during the qPCR or barely detectable transcripts in individual samples in the control or Gremlin1 supplemented conditions, were excluded from the analysis.

Long term culture-initiating cell (LTC-IC) assay

For these experiments, $0.5–1 \times 10^3$ CD34+/CD38−/lin− cord blood cells were placed in microporous collagen-coated 0.4-mm Transwell® inserts in 12- or 6-well plates in medium alone or medium supplemented with BMP-2/7 heterodimer or BMP-4 (10 ng/ml each) or Gremlin1 (50 ng/ml). AFT024 stromal cells (for the limiting dilution assays) were expanded and irradiated as detailed [35]. After 2 or 5 weeks of culture, LTC-IC frequency in cells recovered from the Transwells was determined using a limiting dilution method as previously described [29–31, 36,37] and expressed as a percentage of LTC-IC frequency in the starting population (at day 0).

Assessment of transplantable progenitors

CD34+/CD38−/lin− cord blood cells (10^4/mouse: day 0 condition) or the total progeny of an equivalent number of cells cultured for 2 or 5 weeks in medium alone or medium supplemented with 50 ng/ml Gremlin1 were injected via tail vein into sublethally irradiated 8 week old NOD-scid-IL2Rg^{null} (NSG) mice, together with irradiated CD34− human accessory cells. Ten weeks later, human cell engraftment in the bone marrow was determined by the percentage of human CD45+ cells, using flow cytometry. The proportions of human cells in bone marrow harvested from both femurs and both tibiae were determined by the percentage of human CD45+ cells (using a hCD45-APC antibody) by flow cytometry on a BD FACSAria III flow cytometer. Samples with engraftment of ≥1% were further assessed for myeloid and lymphoid markers (FITC-CD15, PerCP-Cy5.5-CD33, PE-Cy7-CD34, APC-Cy7-CD14, FITC-CD19, and APC-Cy7-CD3) to determine the relative proportions of corresponding lineages derived from engrafted human SRC.

Statistical analysis

Significance of differences in LTC-IC was calculated by an unpaired, two-tailed t-test (GraphPad Prism v5; GraphPad Software, San Diego, CA). Significance of differences in proportions of human cells engrafted in NSG mice was calculated by an unpaired, two-tailed t-test with Welch's correction. Significance of alteration in gene transcript levels was determined by the Wilcoxon matched-pairs signed rank 2-tailed