A Novel Small Molecule E-Selectin Inhibitor GMI-1271 Blocks Adhesion of AML Blasts to E-Selectin and Mobilizes Blood Cells in Nodscid IL2Rgc-/- Mice Engrafted with Human AML

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ABSTRACT

The E-selectin ligand HCELL (hematopoietic cell E-/L-selectin ligand) is expressed by normal hematopoietic stem cells (Merzaban et al Blood 2011) as a functional glycoform of CD44. We observe high level CD44 expression (99%± 1.4%) by blasts from 55 consecutive patients with acute myeloid leukemia (AML) and by putative CD34+CD38-CD123+ leukemia stem cells (LSCs) (99.8% ± 0.6%). The mean fluorescence intensity (MFI) for CD44 expression by AML blasts is one to two logs higher than the MFI for 16 other adhesion receptors. We find that the majority of blasts from patients with AML also express an E-selectin ligand by flow cytometry: >75%% of 22 primary gated blast samples exhibit ≥10% binding of E selectin-IgG chimeric protein with a mean of 22.7% ± 0.17%SD, range 1.8 to 66.2%. We demonstrated that this ligand was HCELL by immunoprecipitation of CD44 from AML cell membranes, followed by staining with HECA 452 antibody that recognizes a functional trisaccharide domain shared by sialyl Lea and sialyl Lex and known to bind to E-selectin. HECA 452 not only detects the functional glycoform of CD44 known as HCELL, a major ligand for E-selectin, but also identifies the human lymphocyte homing receptor CLA (cutaneous lymphocyte antigen). HECA 452 labeled 5 of 6 patient leukemia blast populations, with mean expression 59.0% ± 24.8%. We also observed that HECA 452 antibody labeled CD34+CD38-CD123+ LSCs in addition to leukemic blasts, with a higher percent expression in most cases for the LSCs than the corresponding unfractionated blast population. Moreover, HECA 452 labeled 94% of human AML cells that had been serially engrafted in NODscid IL2Rgc-/- animals, fulfilling the functional definition of LSCs (scid repopulating cells), suggesting that HCELL may be enriched on LSCs . We observe a change in morphology with binding of AML blasts to E-selectin coated plastic, in that they elongate, become more cuboidal and less reflective, in contrast to the non-adherent cells, which remain round and refractile. AML blasts appear to bind to the elongated ends of spindle shaped endothelial cells. The E-selectin-specific inhibitor GMI-1271 here(concentration 20 μM) inhibited adhesion of primary human AML cells to E-selectin by an average of 45.0%± 9.1%SD for all patients. For one patient, AML 035, for example, the percent inhibition with GMI-1271 compared to media control was 33.4%± 15.3%SD, p=0.000018. We demonstrated that a dual inhibitor of E selectin and CXCR4 (GMI-1215) mobilized human AML engrafted in NODscid IL2Rgc-/- mice (Chien et al Abstract 579, ASH 2011), to a greater degree than we observed with CXCR4 inhibitor plerixafor (Chien et al Abstract 1432, ASH 2011) alone (3-4 fold vs. ~2 fold). We now report that the E-selectin-specific inhibitor, GMI-1271 (40mg/kg), mobilizes both human and murine cells in immunodeficient xenograft mice engrafted with human AML: there
is a 2 fold increase in WBC (p=0.00067) and 2 fold increase in human AML cells (p=0.14) at 3 hrs. An initial experiment with a combination of GMI-1271, daunorubicin, and cytarabine demonstrated greater depletion of human AML from the bone marrow (22% as many AML cells) and spleen (31% as many AML cells) than daunorubicin and cytarabine alone. Additional in vivo studies are in progress. We propose that residence in the bone marrow vascular niche may involve E-selectin and that migration of AML blasts involves critical interactions with the vascular endothelium through E-selectin. This interaction between HCELL expressed by AML and E-selectin might represent a new potential therapeutic target for AML.

INTRODUCTION

CD44 plays a pivotal role in the bone marrow homing of leukemia cells. For example, anti-CD44 antibodies prevent engraftment of LSCs in NODscid animals (Jin L et al Nat Med 2006;12:1167-74). Bcr-abl positive leukemia stem cells are dependent on CD44 for homing to the marrow (Krause et al Nat Med 2006; 12: 1175-1180). Nalm6 ALL cell line homes to Eselectin+SDF1+ regions of the bone marrow microvasculature (Sipkins DA et al, Nature 2005, 435: 969-973), dependent on both E selectin and CXCR4.

Moreover, level of CD44 expression confers prognostic significance, in that high level expression of CD44 correlates with leukemic relapse (Quere et al Leukemia 2010), and high level soluble CD44 in hematopoietic stem cell grafts from patients with AML also correlates with increased rate of relapse (Krause DS et al, ArchPatholLabMed 2010;134:1033-38).

When properly glycosylated, CD44 functions as an E-selectin ligand, "HCELL," expressed by normal hematopoietic stem cells (Merzaban et al Blood 118: 1774-1783, 2011).

Lastly, selectins appear to play a role in the interaction of certain cancer cells with their microenvironment. For example, a pan selectin inhibitor disrupts myeloma cell interaction with the bone marrow and sensitized myeloma cells to bortezomib (Azab A Blood 2012; 119:1468-78). Lastly, adhesion of colon carcinoma cells to E selectin led to activation of survival pathways (Porquet et al . BMC Cancer 2011, 11:285-296), implying that disruption might also impair these survival pathways and enhance the efficacy of chemotherapy.

GMI-1271 is a potent new rationally designed small moleule E-selectin antagonist.

PROCEDURES

1. Flow Cytometry Analysis for E-selectin-IgG chimera binding by AML blasts and leukemia stem cells
2. Flow Cytometry Analysis with HECA 452 Antibody Staining
3. Binding of AML blasts to E selectin and blocking by GMI 1271
4. In vitro chemotherapy cytotoxicity of daunorubicin and cytarabine on AML blasts on E selectin with or without GMI 1271
5. In vivo cytotoxicity with combination of chemotherapy and GMI 1271 in NODscid IL2R gc-/- mice engrafted with primary human AML

Method

Flow cytometry analysis of HCELL and E-Selectin

AML cells were stained for 30 minutes at 4°C with CD45-APC-Cy7 to gate on the blast cells, and CD34-APC, CD38-PE-Cy7 and CD123-PE (or CD123-FITC) were used to gate on the AML stem cells. The conjugated anti-CLA-FITC (CLA: Cutaneous Lymphocyte Antigen, HECA-452 clone) antibody and Human E-Selectin/CD62E Fc Chimera-PE were used to assess E-selectin ligand expression. The samples were washed and then fixed with 1% paraformaldehyde. Cells were measured by FACScanto (BD biosciences) and the results were analyzed using Flowjo software. (Tree Star, Inc.)

Procedures for in vivo studies of chemotherapy + GMI 1271 in AML xenograft

Human Subjects

Blood and bone marrow samples were obtained from AML patients by informed consent on a protocol approved by the UW/FHCRC Institutional Review Board.

Animals

Nod-Scid (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) mice were obtained from Jackson Laboratories. They were maintained and bred in an SPF facility at University of Washington, Seattle, WA. Animal care and study protocols were approved by Institutional Animal Care and Use Committee (IACUC) of University of Washington School of Medicine

Engraftment of human AML

Mice received 275 to 300 cGy sublethal total body irradiation by gamma irradiator. Each mouse received 1.3 to 2 million primary human AML mononuclear cells by tail vein injection. We first detect circulating human CD45 positive cells, denoting engraftment by flow cytometry as early as 4-10 weeks.

Inhibitor + chemotherapy treatment

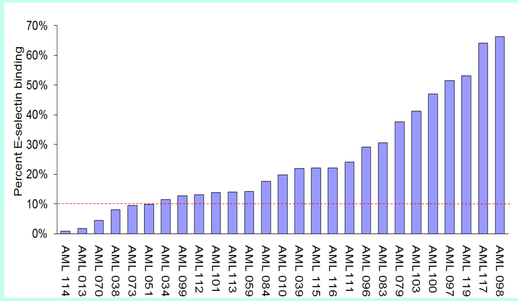
We studied the combination of GMI1271 40mg/kg IP twice a day for three days, cytarabine 300mg/kg IP once a day for 3 days and Daunarubicin 3mg/kg IV single dose. Animals were sacrificed by 12 days after chemotherapy, and assessed for AML in blood, marrow, and spleen.

Flow cytometry analysis of engraftment

Mouse blood, bone marrow (BM) and spleen cells were labeled with human CD45 conjugated APC-H7 and mouse CD45 conjugated PE. After washing, the cells were fixed with 2% paraformaldehyde . Flow cytometry analysis was performed using the FACScanto (BD Biosciences). The results were analyzed using Flowjo software (Tree Star, Inc).

RESULTS

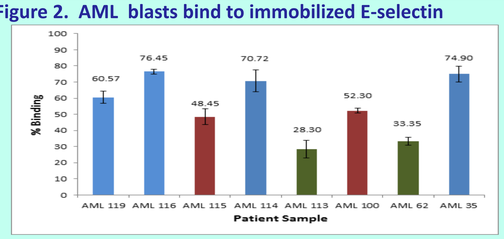
Figure 1. Flow Cytometry for E selectin-Ig binding



Blasts from 85% of AML patients bind E selectin-Ig chimeric molecule (≥10%), mean 25.4% ± 0.2%, consistent with expression of an E selectin ligand.

RESULTS

Figure 2. AML blasts bind to immobilized E-selectin



Adhesion of leukemia cells to E-selectin ranged from 28.3% to 76.45% with an average of 55.6 ± 18.4%. High adhesion samples (AMLs 119, 116, 114, 35) are shown in blue, intermediate adhesion samples (AMLs 115, 100) in red, and low adhesion samples (AMLs 113, 62) in green.

Figure 3. Adhesion of AML to E-selectin with or without GMI 1271 ~~Inhibitor~~

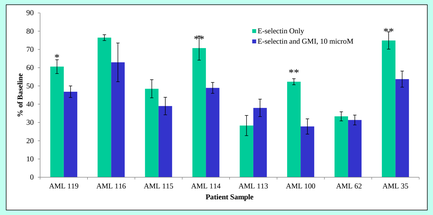
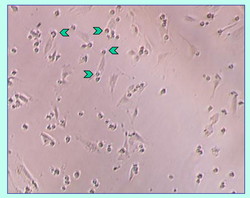


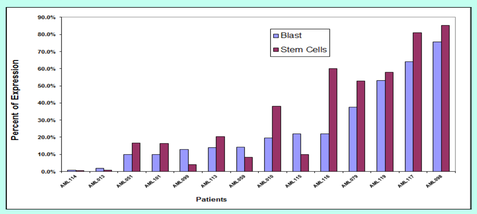
Figure 3. Seven of 8 patient samples showed reduced attachment to E-selectin when blocked with GMI 1271. Patient samples with significant differences between blocked and control groups are denoted by asterisks.

Figure 4. Photomicrograph of AML associating with HUVEC



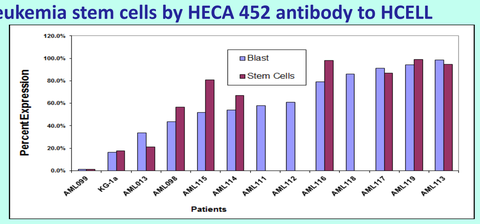
AML blasts appear to be associating at end of spindle shaped HUVEC (human umbilical vein endothelial cells. Binding is blocked by GMI 1271.

Figure 5. Flow cytometry analysis of binding of E selectin-Ig chimera to AML blasts and CD34+CD38-CD123+ leukemia stem cells



Putative leukemia stem cells (LSCs) express an E selectin ligand. In most cases, there is higher expression by the LSCs (p=0.03)

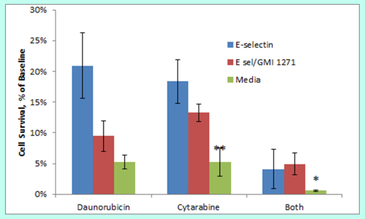
Figure 6. Flow cytometry staining of AML blasts and leukemia stem cells by HECA 452 antibody to HCELL



Most primary AML blasts and leukemia stem cells exhibit a high percent of cells positive for CLA.

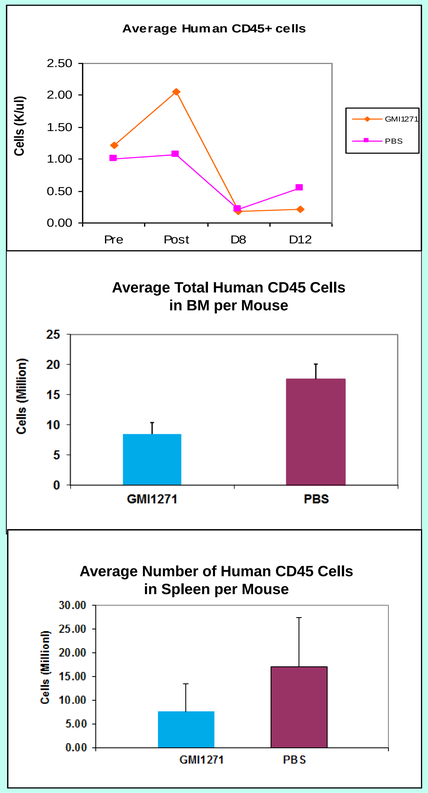
RESULTS

Figure 7. Example of E selectin conferring chemotherapy drug resistance in vitro



There was variable chemotherapy protection afforded by binding to E-selectin for different primary AML samples. In this case, cells plated on E-selectin exhibited improved survival than cells plated on media, and GMI-1271 reversed this E-selectin-mediated drug resistance.

Figure 8. Cytotoxicity of Combination Chemotherapy + GMI 1271 in NODscid IL2Rgc $^{-/-}$(NSG) xenograft (w/primary AML)



SUMMARY

1. The majority of patient AML blasts express a functional E-selectin ligand.
2. Putative leukemia stem cells also express an E-selectin ligand.
3. E selectin-specific inhibitor GMI-1271 inhibits binding of AML to E selectin.
4. Some patient samples exhibit protection from cytotoxic chemotherapy when bound to E-selectin that is reversed by GMI-1271.
5. The combination of GMI-1271 /daunorubicin/araC reduces bone marrow and spleen involvement by AML in AML xenografts to a greater extent than dauno/araC alone.

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