Engraftment of Human Primary Acute Myeloid Leukemia Defined by Integrated Genetic Profiling in NOD/SCID/IL2rnull Mice for Preclinical Ceramide-Based Therapeutic Evaluation

Brian M Barth1,2*, Nichole R Keasey1,2, Xujung Wang1,2, Srima Shanmugavelandy2, Raajit Rampal3,5, Todd Hricik4,5, Myles C Cabot6, Mark Kester7, Hong-Gang Wang4,7,8, Leonard D Shultz2, Martin S Tallman4, Ross L Levine4,5; Thomas P Loughran Jr.1,2 and David F Claxton4,5

1Department of Medicine, Penn State College of Medicine, Hershey, USA
2Penn State Hershey Cancer Institute, Penn State College of Medicine, Hershey, USA
3Department of Pharmacology, Penn State College of Medicine, Hershey, USA
4Leukemia Service, Department of Medicine, Memorial Sloan-Kettering Cancer Center, USA
5Human Oncology and Pathogenesis Program, Memorial Sloan-Kettering Cancer Center, USA
6Department of Biochemistry and Molecular Biology, East Carolina University, Greenville, USA
7University of Virginia Cancer Center, Charlottesville, USA
8Department of Pediatrics, Penn State College of Medicine, Hershey, USA
9The Jackson Laboratory, Bar Harbor, USA

*Corresponding author: Dr. Brian M Barth, Department of Medicine, Division of Hematology and Oncology, Penn State Hershey Cancer Institute, Penn State College of Medicine, 500 University Drive, PO Box 850, CH46 Hershey, PA 17033, USA, Tel: 717-531-0003 (289457); E-mail: bmb14@psu.edu

Rec date: Apr 4, 2014; Acc date: Jul 10, 2014; Pub date: Jul 25, 2014

Copyright: © 2014 Barth BM et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Abstract

Acute Myeloid Leukemia (AML) is a highly heterogeneous and poor prognosis disease with few available therapeutic options. Novel advances are urgently needed, however effective models to test experimental therapeutics have been lacking. Recently, NOD/SCID/IL2rnull (NSG) mice were shown to engraft primary human AML in a manner that recapitulated the natural disease and its progression. Additionally, integrated genomic profiling was used to refine risk stratification of AML. In this study, we demonstrated the engraftment of molecularly defined primary AML in NSG mice. We showed that AML that express DNMT3A mutations, which predict for adverse outcome, engrafted with exceptional efficacy. Lastly, we demonstrated that human AML-engrafted NSG mice can be effectively used to study novel ceramide-based therapeutics. Ceramide is a bioactive sphingolipid that inherently dependent on the usefulness of preclinical animal models. In the case of AML, murine models have proved disappointing for therapeutic development due primarily to the inability to replicate the human disease. The engraftment of human AML stem cells within the bone marrow of sublethal-irradiated NOD/SCID/IL2rnull (NSG) mice has recently been reported, with these mice developing a more efficient disease compared with other immunodeficient strains [4-9]. Of particular note, enhanced engraftment of human AML with FLT3 mutations was reported as compared with wild-type FLT3-containing human AML [7]. Improvements on engraftment efficiency within this model have focused on transgenic expression of genes expressing human cytokines and growth factors [10]. Unfortunately, reports of the utility of this model for novel therapeutic testing are limited [6,11,12]. In the present report, we have used integrated mutational profiling to refine the risk stratification of human AML samples which successfully engrafted into NSG mice. We describe robust engraftment activity against specific molecularly defined and risk stratified AML.

Keywords: Acute myeloid leukemia; Integrated genetic profiling; DNMT3A; NSG mouse; Ceramide; Tamoxifen; Nanoliposome

Introduction

Acute Myeloid Leukemia (AML) is a growing public health problem in the United States with few available therapeutic options. AML is a highly heterogeneous disease or group of diseases. The 2008 WHO classification is accepted for clinical AML classification and incorporates known karyotypic data of prognostic importance but fails to predict outcome for many cytogenetically normal AML patients [1,2]. Recently, specific mutations were identified that predict AML outcome and improve risk stratification independent of historically recognized risk factors [2,3]. We had previously shown that this schema included mutations in TET2, DNMT3A, ASXL1, PHF6, and MLL, which predicted for adverse outcome, as well as patients with coincident mutations in NPM1 and in IDH1/2, which were associated with favorable outcome [2].

Novel advances for the treatment of AML are urgently needed, however appropriate models to test experimental therapeutics have been lacking. The translation of laboratory studies to clinical studies is inherently dependent on the usefulness of preclinical animal models. In the case of AML, murine models have proved disappointing for therapeutic development due primarily to the inability to replicate the human disease. The engraftment of human AML stem cells within the bone marrow of sublethal-irradiated NOD/SCID/IL2rnull (NSG) mice has recently been reported, with these mice developing a more efficient disease compared with other immunodeficient strains [4-9]. Of particular note, enhanced engraftment of human AML with FLT3 mutations was reported as compared with wild-type FLT3-containing human AML [7]. Improvements on engraftment efficiency within this model have focused on transgenic expression of genes expressing human cytokines and growth factors [10]. Unfortunately, reports of the utility of this model for novel therapeutic testing are limited [6,11,12]. In the present report, we have used integrated mutational profiling to refine the risk stratification of human AML samples which successfully engrafted into NSG mice. We describe robust engraftment...
efficiency, with the novel finding that cases harboring DNMT3A mutations engrafted with exceptional efficacy. Finally, we provide proof of concept that the NSG model can be used to study the anti-AML efficacy of nanoliposomal C6-ceramide and nanoliposomal tamoxifen, which are novel ceramide-based therapeutics developed by our laboratories [13,14].

Materials and Methods

Integrated Mutational Profiling

Human AML samples were collected with informed consent and approval from the Penn State College of Medicine Institutional Review Board. Mononuclear cells were prepared by Ficoll separation, and cryopreserved prior to use. Genomic DNA was collected by extraction using a Qiagen DNeasy Kit according to the manufacturer’s recommendations. Mutational profiling was performed as previously described in detail [2]. Briefly, mutational analysis of the entire coding regions of TET2, ASXL1, DNMT3A, PHF6, WT1, TP53, EZH2, RUNX1, and PTEN and of coding exons of FLT3, HRAS, KRAS, NRAS, KIT, IDH1, and IDH2 with known somatic mutations was performed using PCR amplification with the RainDance multiplex microdroplet PCR algorithm followed by Illumina HiSeq sequencing to ensure high coverage in the entire target region.

Nanoliposome Preparation

Nanoliposomes were prepared as previously described [14-21]. Briefly, lipids dissolved in chloroform, were combined in specific molar ratios, dried to a film under a stream of nitrogen, and then hydrated by addition of 0.9% NaCl. Solutions were sealed, heated at 60°C for 60 minutes, and subjected to vortex mixing and sonicated until light no longer diffracted through the suspensions. The lipid vesicle-containing solutions were quickly extruded at 60°C by passing the solutions 10 times through 100 nm polycarbonate filters using an Avanti Mini-Extruder (Avanti Polar Lipids, Alabaster, AL). Nanoliposomal size and a neutral charge were validated using a Malvern Zetasizer Nano ZS at 25°C. Nanoliposome solutions were stored at room temperature until use.

NSG Engraftment and Treatment

Briefly, adult NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ (NSG) mice (8-10 wks old) received 250 cGy of sublethal total body irradiation, followed by tail vein injection of primary human AML cells. Cryopreserved cells were injected intravenously at doses of 0.5-10x10^6 viable mononuclear cells containing 80-90% leukemic blasts. Engraftment was monitored in by flow cytometry in 20 μl samples of tail vein peripheral blood with anti-human CD13, CD33, HLA-DR, and CD45 antibodies at 1-4 week intervals. Engraftment was defined as >0.5% cells expressing human CD45 and one other human marker. For subsequent transplantation, mice were euthanized and bone marrow mononuclear cells were harvested from tibiae and femurs and injected into recipients. For therapeutic evaluation, C6-ceramide nanoliposomes or tamoxifen nanoliposomes were administered via tail vein injection as previously described three times per week for up to two months [14-20]. Treatments were commenced following verification of engraftment in peripheral blood (>0.5%), and human AML burden was routinely monitored in the peripheral blood. Specifically, 20 μl blood collected by pricking the tail vein was added in precise volume to a solution of antibodies and counting beads. Blood was subsequently evaluated by flow cytometry using either a BD Canto II or BD LSR II flow cytometer. The Penn State College of Medicine Institutional Animal Care and Use Committee approved all animal procedures.

Results and Discussion

Herein, we report that AML isolated from the peripheral blood of human patients was successfully engrafted into sublethally irradiated NSG mice. Engraftment of human AML cells in NSG mice was defined by the prevalence of cells with both CD13 and CD33, or either in combination with one other human marker (HLA-DR, CD45) when monitored by flow cytometry of peripheral blood at various time points or bone marrow or spleen at necropsy (Figure 1 a-d). We have also evaluated CD19 or CD3 in select cases to distinguish normal hematopoietic cells (data not shown), however co-expression of lymphoid markers with CD33 or CD19 identifies AML with multilineage dysplasia. Overall, we observed a primary engraftment efficiency of 81% (31/38 mice) for a total engraftment of 13/14 human AML cases evaluated (Table 1).

Figure 1: Monitoring the therapeutic efficacy of C6-ceramide and tamoxifen nanoliposomes in NSG mice engrafted with human AML. NSG mice were given sublethal total body irradiation prior to engraftment with human AML via tail vein injection. Engraftment was evaluated by flow cytometry of the peripheral blood (A), and bone marrow (B), using anti-human CD13, CD33, HLA-DR, and CD45 antibodies. (C) Treatment was initiated following engraftment confirmation, and the anti-AML efficacy of C6-ceramide nanoliposomes (Lip-C6), or control nanoliposomes (Lip-Ghost), was evaluated using NSG mice engrafted with a poor prognosis human AML sample (inv3, -7), and leukemia burden was routinely monitored by analysis of blood collected from tail vein prick. *p<0.001, 2-way ANOVA, n=4 mice per group, error bars represent standard error of the mean. (D) Human AML engraftment was assessed by flow cytometry of spleen and bone marrow preparations following necropsy at day 98. Included is a comparison with tamoxifen nanoliposomes (Lip-Tam), as well as nanoliposomes loaded with both C6-ceramide and tamoxifen (Lip-C6/Tam) (*p=0.0317, unpaired t-test comparing Lip-Ghost and Lip-Tam).
The utility of NSG mice engrafted with primary human AML has been limited. Initially, Saito et al. used Ara-C to define a chemotherapy resistant AML stem cell population [6], and recently used the NSG model to evaluate RK-20449, a novel hematopoietic cell kinase-targeted compound identified through drug screening [11]. In another example, Herrmann et al. used the NSG model to evaluate heat shock protein inhibitors, but did so by treating patient AML in vitro prior to engraftment [12]. In our study, we evaluated the preclinical efficacy of nanoliposomal C6-ceramide and nanoliposomal tamoxifen, as well as a combinatorial ceramide/tamoxifen nanoliposomal formulation, in NSG mice engrafted with a poor prognosis primary human AML (case 329: inv3, -7). In this study we were able to readily monitor the leukemia burden in mice by analyzing tail vein blood, and noted that nanoliposomal C6-ceramide blunted the exponential growth of leukemia (Figure 1c). Additionally, at necropsy we were able to note a significant decrease in the leukemia burden in the spleen, but not the bone marrow, of mice treated systemically with nanoliposomal tamoxifen, but not other agents (Figure 1d). This study confirmed that NSG mice engrafted with human AML can be successfully used for preclinical evaluation of novel experimental therapeutics.

Various therapeutics induce ceramide generation, a bioactive sphingolipid that regulates cellular stress and death, while metabolic pathways to eliminate ceramide have gained notoriety as mechanisms of therapy resistance [13,23,24]. We have shown that tamoxifen is a potent inducer of ceramide accumulation through a mechanism preventing P-gp mediated ceramide glycosylation at the Golgi membrane [13,14]. We have demonstrated robust in vivo anticancer efficacy for nanoliposomal ceramide in preclinical models of breast cancer, pancreatic cancer, melanoma, LGL leukemia, and hepatocellular carcinoma, and recently demonstrated its combinatorial efficacy with nanoliposomal tamoxifen in an in vivo model of colorectal cancer [13,14]. Our present study revealed a modest yet significant efficacy for nanoliposomal C6-ceramide and nanoliposomal tamoxifen as anti-AML therapeutics that can reduce the blood or spleen leukemia burden, respectively. Importantly, the NSG model allowed the evaluation of a poor prognosis human AML effectively reconstituted in the murine host. It is noteworthy that the combination of nanoliposomal C6-ceramide and nanoliposomal tamoxifen did not exert therapeutic efficacy in this particular AML case (Figure 1d). This may be indicative of a further need to evaluate how the underlying biology in specific subtypes of AML relates to sphingolipid metabolism. Too many generalizations about the potential efficacy of experimental therapeutics such as nanoliposomal C6-ceramide and nanoliposomal tamoxifen or their combination, may focus on selective targeting strategies to improve their overall therapeutic efficacy, and may focus on evaluating efficacy in molecularly specific AML subtypes. Furthermore, the expansion of human AML in the bone marrow of NSG mice can allow sufficient cell numbers to perform post-treatment analysis to evaluate changes in the sphingolipidome associated with ceramide-based therapeutics. Altogether, this work demonstrates the potential to explore in vivo drug sensitivity of molecularly defined human AML in the NSG mouse. This model is likely to prove valuable in the study of novel agents with therapeutic anti-AML activity including ceramide-based therapeutics.
This study was funded in part by the Penn State University Kiesendahl Family Endowed Leukemia Research Fund, the Kenneth Noel Memorial Fund, PA Tobacco Settlement funds, NIH grant CA171983 (B.M.B., M.C.C., M.K., H.G.W., L.D.S., M.S.T., R.L.L., T.P.L., AND D.F.C.) and NIH grant CA034196 (L.D.S.). The Penn State Research Foundation has licensed ceramide nanoliposomes, and other nonliposomal technology, to Keystone Nano, Inc. (State College, PA). M.K. is Chief Medical Officer of Keystone Nano, Inc.

References