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BRIEF COMMUNICATIONS

Acute myeloid leukemia xenograft success prediction: Saving time

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Xenograft assay allows functional analysis of leukemia-initiating cells of acute myeloid leukemia primary samples. However, 40% of samples derived from patients with better outcomes fail to engraft in immunodeficient mouse recipients when conventional protocols are followed. At diagnosis, the engraftment of intermediate-risk group samples cannot be anticipated. In this study, we decided to further explore the reasons for xenograft success and failure. No differences in extracellular phenotype, apoptosis, or cell cycle profile could distinguish samples that engraft (engrafter [E]) from samples that do not engraft (nonengrafter [NE]) in NSG mice. In addition, ex vivo long-term culture assay revealed, after 5 weeks, a lower content of leukemic-LTC-initiating cells in the NE samples associated with a lower expansion rate capacity. One-week co-cultures with mesenchymal or osteoblastic or endothelial cells did not influence the proliferation rate, suggesting that E and NE samples are genuinely rapidly or slowly expanding independent of external cue. Engraftment success for some NE samples was consistently observed in recipient mice analyzed 6 months later than the conventional 3-month period. Eventually we implemented a flow cytometry-based assay, which allowed us to predict, in 1 week, the fast or delayed engraftment potential of a noncharacterized acute myeloid leukemia sample. This approach will be especially useful in selecting intermediate-risk-group patient samples and restricting the experimental duration to a 3-month period and, eventually, in reducing the number of animals and the cost and effort of unnecessary xenograft failures.

The in vivo xenotransplantation assay in NOD/SCID IL-2Rγ common chain null (NSG) mice is currently the model most frequently used to study the biology of leukemia-initiating cells (LICs); however, a substantial proportion of samples from patients with acute myeloid leukemia (AML) with a good prognosis fail to engraft in mice. Other newly described humanized mouse models such as NSG-SG3M and MISRTG mice might improve such sample engraftment [1]. Yet, we recently evidenced the extinction of myelodysplastic syndrome propagating cells (MDS-PCs) using NSG-SG3M mice, which suggests that human cytokine stimulation might exhaust the LIC compartment of particular leukemias [2]. Alternatively, we found that subcutaneous implantation of gelatin sponges seeded with human stromal cells allows engraftment of good-risk AML in NSG mice. However, as observed by others using subcutaneous humanized ossicles, these ectopic leukemic grafts do not invade recipient bone marrow [3–5]. Because all these models are either not fully characterized or not fully available, the straightforward intravenous NSG model is still the most commonly used model. Here we further investigated xenograft failure in this model and developed a

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flow cytometry-based assay that allow prediction of the xenograft potential of a noncharacterized AML sample.

Methods

Cells

AML cells were obtained after receipt of informed consent from St Bartholomew's Hospital. Details of the patient samples are listed in Supplementary Table E1 (online only, available at www.exphem.org). Co-culture experiments were previously described [6]. AML samples were collected at diagnosis, and mononucleated cells were isolated within 24 hours after collection by Ficoll-Paque Plus density gradient (GE Healthcare, France). Cord blood (CB) cells were obtained after receipt of informed consent from the Royal Free Hospital (UK). Both AML and CB sample collections were approved by the East London ethical committee and in accordance with the Declaration of Helsinki. Three to 5 different CB samples were pooled, and mononuclear cells were obtained by density centrifugation. Lineage markers expressing cells were depleted using StemSep columns and human progenitor enrichment cocktail (StemCell Technologies, Vancouver, BC, Canada). $CD34^+CD38^-$ cells (hematopoietic stem progenitor cells [HSPCs]) and $CD34^+CD38^+$ cells (hematopoietic progenitor cells [HPCs]) were sorted on a MoFlo cell sorter (DakoCytomation Colorado, Fort Collins, CO, USA) or a BD FACS Aria (BD Biosciences, UK). Gates were set up to exclude nonviable cells and debris. Briefly, lineage-depleted recovered cells were washed twice and stained with anti-CD34 Percp, anti-CD38 PE-cy7, AlexaFluor647-conjugated Annexin-V (Invitrogen), and DAPI (4',6-diamidino-2-phenylindole). The purity of sorted fractions was assessed to ensure the sort quality. The stromal cell line mesenchymal MS-5 and the human osteosarcoma cell line SaOS-2 were obtained from the DSMZ cell bank (Braunschweig Germany) and maintained in Iscove's modified Dulbecco's medium (IMDM) containing 10% fetal calf serum (FCS) + 2 mmol/L L-glutamine or in McCoy's 5a medium containing 15% FCS + 2 mmol/L L-glutamine, respectively. Human umbilical vein endothelial cells (HUVECs) obtained from Lonza were propagated in endothelial growth medium-2, EGM-2-MV (Lonza, UK) in culture dishes coated with type I collagen (StemCell Technologies). MS-5, SaOS-2, and HUVEC feeders were cultured in their respective media and subcultured when reaching 80% confluence. Sca-1, CD56, and CD31 were identified as specific markers for 100% of MS-5, SaOS-2, and HUVEC, respectively, and used for feeder exclusion in fluorescence-activated cell sorting (FACS) analysis. All three antibodies were from BD Pharmingen (Oxford Science Park, UK).

Adoptive transfer of human hematopoietic cells in immunodeficient mice

NOD/SCID (NS) and NSG mice were a kind gift of Dr. Leonard Shultz. All animal experiments were performed in accordance with Home Office and CRUK guidelines. Adult NS or NSG mice were injected intravenously with 10^7 T-depleted mononuclear AML cells. In the current study, we define nonengrafter [NE] samples as samples for which 10^7 $CD3^+$-depleted AML MNCs injected cells were not able to engraft at a detectable level (cutoff: 0.1%) of human myeloid-only leukemic population $CD45^+CD33^+CD19^-$ and murine $CD45^-$, 12 weeks after injection into NSG mice. For newborn xenograft, 2.5 to 10×10^6 AML cells were injected into day 2 neonate NSG mice via an intrahepatic (IH) or facial (FV) vein. For some engrafter (E) samples, LIC and non-LIC phenotypes were functionally defined by xenograft experiments with sorted subpopulations (Supplementary Figure E1, online only, available at www.exphem.org). Mouse bone marrow cells were collected and analyzed by flow cytometry as detailed previously [7,8].

Long-term culture

Co-culture experiments were performed as previously described [9,10] as bulk culture or using a limiting dilution analysis (LDA) both on confluent monolayers of MS-5, supplemented with recombinant human interleukin (IL)-3, granulocyte colony-stimulating factor (G-CSF), and TPO (MS-5 + 3GT) (20 ng/ml each; Peprotech, London, U.K., http://www.peprotech.com) in MyeloCult H5100 (StemCell Technologies, Vancouver, BC, Canada, http://www.stemcell.com). Cells were cultured at 37°C in 5% CO_2-humidified incubators. Cells were plated in 20 replicates in 96-well microplates containing confluent MS-5 monolayers. Half the medium was done twice a week without disrupting the established feeders. After 5 weeks, LTC medium was replaced by methylcellulose H4435 (StemCell Technologies). After an additional 2 weeks, each well was scored as negative if no colonies were present. To determine the frequency of leukemia long-term culture-initiating cells (L-LTC-ICs), LDA was calculated using LCalc software (StemCell Technologies) according to Poisson statistics and the method of maximum likelihood.

Fluorescence dilution factor

AML cells (1 to 10×10^5) were thawed and stained with 0.8 µmol/L carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen, UK) for 10 min at 37°C in phosphate-buffered saline (PBS). Cells were washed twice and incubated in duplicate on pre-established confluent MS-5 (or SAOS-2 or HUVEC) layers at 37°C in Myelocult medium (StemCell Technologies) without cytokine supplementation. Cells were incubated 18 hours before assessing the initial input CFSE fluorescence intensity to allow for the turnover of CFSE-labeled proteins to stabilize [11]. After 18 hours and 1 week, wells were harvested by trypsinization and stained with anti-Sca-1-PE (for MS-5 staining), or anti-CD56-PE (for SaOS-2 staining), or anti-CD31-PE (for HUVEC staining) and anti-CD45-APC-Cy7 antibodies (BD Pharmingen). The CFSE median fluorescence index (MFI) was measured by FACS at 18 hours and day 7 on viable (Annexin V and DAPI negative) human hematopoietic cells (CD45 positive and Sca-1 negative). FDF was defined as the ratio of the 18-hour CFSE MFI to the 1-week CFSE MFI. AML heterogeneity evidenced through FACS scattered light measurements meant that the width of the labeled input population exceeded the limits for peak resolution, even under optimal instrument conditions [12,13]. As we reported previously [10], larger cells labeled more brightly with CFSE compared with smaller blasts within the same sample. Consequently, as the cells divide, the width of each division peak overlaps heavily with previous and subsequent peaks, preventing accurate peak resolution. Instead, we defined the fluorescence dilution factor (FDF) as the ratio of the 18-hour CFSE MFI to that of the D7 CFSE MFI. The CFSE MFI was measured on viable (Annexin V-Alexa Fluor 647 and DAPI negative) human hematopoietic cells (CD45-APC-Cy7 positive and Sca-1-PE or CD56-PE or CD31-PE negative, excluding residual normal lymphocytes $CD45^{high}/SCC^{low}$ for all analyses. At day 7, the same procedure was applied. Cytometric calibration was controlled using CountBright beads.