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Bone marrow microenvironment modulation of acute lymphoblastic leukemia phenotype

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Acute lymphoblastic leukemia (ALL) treatment regimens have dramatically improved the survival of ALL patients. However, chemoresistant minimal residual disease that persists following cessation of therapy contributes to aggressive relapse. The bone marrow microenvironment (BMM) is an established "site of sanctuary" for ALL, as well as myeloid-lineage hematopoietic disease, with signals in this unique anatomic location contributing to drug resistance. Several models have been developed to recapitulate the interactions between the BMM and ALL cells. However, many in vitro models fail to accurately reflect the level of protection afforded to the most resistant subset of leukemic cells during coculture with BMM elements. Preclinical in vivo models have advantages, but can be costly, and are often not fully informed by optimal in vitro studies. We describe an innovative extension of 2-D coculture wherein ALL cells uniquely interact with bone marrow-derived stromal cells. Tumor cells in this model bury beneath primary human bone marrow-derived stromal cells or osteoblasts, termed "phase dim" ALL, and exhibit a unique phenotype characterized by altered metabolism, distinct protein expression profiles, increased quiescence, and pronounced chemotherapy resistance. Investigation focused on the phase dim subpopulation may more efficiently inform preclinical design and investigation of the minimal residual disease and relapse that arise from BMM-supported leukemic tumor cells.

The bone marrow microenvironment (BMM) is a well-established "site of sanctuary" in a host of malignancies, with the most common being of hematopoietic origin [1–8]. In leukemia, the BMM serves as the site of initiation and progression of disease. The BMM is also the most common site of relapse, where leukemic cells respond to signals that are critical for the support of "healthy" steady-state hematopoiesis [2,9,10]. Quiescence, metabolism, and survival pathways are all influenced by the BMM and are pathways known to be co-opted by leukemic cells in the marrow niche to promote treatment resistance [5,7,11]. Studies from many laboratories have furthered our understanding of the interplay between leukemic cells and the BMM; however, relapse of disease continues to be a clinical challenge.

A number of models have been employed to recapitulate the interactions between the bone marrow niche and leukemic cells. In vivo murine models have provided insight and have become standard preclinical models in which to test novel therapeutic strategies [12–14]. Although in vivo models define the gold standard, they are labor intensive, time consuming, and costly to test hypotheses related to relapse

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of disease. Also, although the BMM can be effectively imaged during disease progression or treatment response, sequential sampling of tumor recovered from the niche is achievable only on termination of experiments, resulting in evaluation of snapshots in time. Often, ongoing analyses are limited to peripheral circulating tumor that does not reflect the most treatment-resistant subpopulation of interest. Standard 2-D in vitro models, while lacking the complexity of the in vivo microenvironment, provide an alternative means to interrogate tumor interactions with the microenvironment. Several groups have reported that 2-D coculture with primary human bone marrow stromal cells (BMSCs) and osteoblasts (HOBs) protect human leukemic cells from chemotherapy-induced death [2,6,8,11,15]. However, standard in vitro models lack the ability to predict long-term survival of subsets of resistant leukemic cells and, as a esult, are not ideal for evaluation of mechanisms that underlie chemoresistant minimal residual disease (MRD).

Studies, including coculture of healthy hematopoietic stem cells with mesenchymal stromal cells (MSCs), have revealed that coculture models exhibit a more dynamic nature than was previously appreciated. Hematopoietic cells interacted with MSCs in three distinct spatial compartments [16]. The subpopulations included uniquely identifiable suspended, phase bright, or phase dim tumor cells when evaluated by light microscopy. Differences in the hematopoietic stem cell phenotype correlated with location of the hematopoietic cell relative to adherent MSCs. Of particular relevance to the current study was the observation that the phase dim population of hematopoietic cells that were buried beneath the MSC monolayer was immature and quiescent, two characteristics that have been associated with chemotherapy resistance [16,17]. In addition, it has previously been described that tumor cells closely associated with BMSC or HOB niches in vivo are more resistant to chemotherapy-induced apoptosis [11,18].

On the basis of previous works we sought to determine whether B-lineage acute lymphoblastic leukemia (ALL) cells, which share many characteristics with their healthy pre- and pro-B-cell counterparts, would localize to distinct compartments of BMSC or HOB coculture, resulting in distinct subpopulations for investigation of therapeutic resistance. We report that ALL cells recovered from the PD population of coculture are phenotypically distinct and exhibit many characteristics of refractory disease described in vivo. PD-derived tumor cells are resistant to therapy, with a survival approximating that of tumor cells not exposed to cytotoxic agents. When compared with the other subpopulations recovered from the same coculture, PD leukemic cells, in addition to their marked survival during chemotherapy exposure, were characterized by increased quiescence and elevated glycolytic activity. Our observations suggest that a biologically relevant model of minimal residual disease can be used in vitro that benefits from the inclusion of relevant human-derived BMM constituents and targeted evaluation of the most resistant component of ALL. The PD leukemic cells in this model lend themselves to more rigorous drug screening than can be achieved when total leukemic populations are evaluated. Importantly, this novel approach of focus on the PD tumor cells may also more efficiently inform preclinical design to investigate MRD and relapse, with specific consideration of resistant subpopulations supported by the BMM.

Methods

Cell lines and culture conditions

Bcr;Abl (Ph+) lymphoblastic cell lines Tom-1 (DSMZ-ACC 578), Nalm-27 (Fujisaki Cancer Center), Nalm-30 (Fujisaki Cancer Center), Sup-B15 (ATCC-CRL-1929), (Ph-) REH (ATCC-CRL-8286) and Nalm-6 (ATCC-CRL-1567) were used. Deidentified primary BMSCs were provided by the Mary Babb Randolph Cancer Center (MBRCC) Biospecimen Processing Core and the West Virginia University Department of Pathology Tissue Bank. BMSC cultures were established as previously described [19]. HOBs were purchased (PromoCell, Heidelberg, Germany) and cultured according to the supplier's recommendations. Cocultures were established by seeding leukemic cells onto 80%–90% confluent BMSC or HOB monolayers. Cultures were fed every 4 days, and tumor cells collected for inclusion in experiments with remaining leukemic cells moved to new primary BMSC or HOB adherent layers every 12 days. Cultures were maintained in 5% O_2 to model normal bone marrow oxygen tension, reported to range from 1% to 7% [20–22]. Suspended (S) leukemic cells floating freely in the medium were removed by gentle pipetting. Phase bright (PB) tumor cells, which were loosely adherent to the top of BMSCs or HOBs, were harvested by vigorous pipetting. Phase dim (PD) leukemic cells that were buried firmly beneath adherent BMSCs or HOBs were recovered by trypsinization of the adherent layer and PD tumor. The S, PB, and PD tumor populations were separated from BMSCs/HOBs by size exclusion with G10 Sephadex (Sigma, St Louis, MO) column separation [23,24].

Microscopy

Phase contrast images were acquired using a Leica DMIL LED microscope and processed by Leica application suite Version 4.0 software (Buffalo Grove, IL). Confocal images were acquired using an upright LSM 510 Zeiss microscope and processed using Zen2009 software (Thornwood, NY). Fluorescence intensity for image acquisition was altered only when fluorescence intensities were not compared between samples.

Subpopulation tracking

The three ALL subpopulations were isolated from coculture as described above. Each subpopulation (S, PB, and PD) was individually stained with CellTracker Green, CellTracker Violet, or CellTracker Deep Red (Life Technologies, Grand Island, NY) following the manufacturer's protocol. An equal number of cells from each population were combined and cultured on coverslips with confluent BMSCs or HOBs for 1, 4 and 48 hours. Coverslips were extensively washed with phosphate-buffered saline to