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Novel Strategy for Successful Long-Term Hematopoietic Recovery after Transplanting a Limited Number of Hematopoietic Stem/Progenitor Cells

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Various investigators have attempted to overcome the shortage of available hematopoietic stem/progenitor cells (HSPCs) by facilitating their engraftment after transplantation. Preconditioning of HSPCs with the granulocyte-derived cationic peptide LL-37 has been suggested as a useful strategy to facilitate engraftment of transplanted cells by enhancing their responsiveness to CXCL12. In this study, we evaluated whether LL-37 preconditioning is acceptable for clinical application. We found that the effect of LL-37 preconditioning was specific to clonogenic cells and was mediated specifically by increased calcium influx with the activation of downstream signaling through mammalian target of rapamycin complex 1 (mTORC1). Because hyper-activation of mTORC1 and the disruption of 5′ adenosine monophosphate-activated protein kinase (AMPK) are known to deplete HSPC pools, we compared the repopulation capacity of HSPCs preconditioned with LL-37 and those preconditioned with AMPK activator (AICAR). In vivo competitive repopulation experiments revealed that LL-37 preconditioning impairs long-term repopulation of transplanted HSPCs, suggesting that this strategy might not acceptable for clinical applications in which long-term repopulation capacity is a prerequisite. AICAR preconditioning dramatically enhanced the long-term repopulation of transplanted HSPCs, however. Taken together, these results suggest that future strategies to ensure successful transplantation outcomes should focus on protecting HSPCs from various stimuli during their homing to the bone marrow niches rather than activating them before transplantation.

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INTRODUCTION

Transplantation of hematopoietic stem/progenitor cells (HSPCs) is used to treat patients with malignant and nonmalignant disorders by reconstituting bone marrow (BM) with healthy stem cells. The current strategy to promote successful hematopoietic recovery after transplantation is to infuse greater numbers of HSPCs; however, the number of cells available for transplantation is limited in most cases. Although the expansion of HSPCs through incubation with various factors has been attempted to overcome the shortage of available sources [1-4], in vitro expanded cells usually show significantly reduced ability to produce long-term hematopoietic reconstitution after transplantation [5,6].

The interaction between CXCL12 and its receptor CXCR4 expressed on HSPCs has been implicated as a primary axis governing HSPC homing and engraftment after transplantation [7]. Therefore, a number of investigators have explored strategies to facilitate HSPC engraftment by enhancing the responsiveness of HSPCs to CXCL12. Recent progress has highlighted the physiological significance of some compounds in leukapheresis products, including the complement cascade cleavage fragment C3a [8] and the granulocyte-derived cationic peptide LL-37 [9], which enhance the responsiveness of HSPCs to CXCL12 in vitro and in vivo. The preconditioning of HSPCs with these components should be a useful approach to solving the HSPC shortage problem [10].

It should be noted, however, that mobilized peripheral blood (PB)–derived HSPCs, which provide rapid reconstitution like C3a- and LL-37–preconditioned HSPCs, have shown

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impaired long-term repopulation in an animal model [11]. We cannot rule out the possibility that the preconditioning strategy may impair the long-term repopulation capacity of transplanted HSPCs, because those components are present in the plasma of normal subjects and patients awaiting transplantation.

HSPCs are usually in a quiescent state and adapt to the hypoxic microenvironment by utilizing glycolysis rather than mitochondrial oxidative phosphorylation in BM niches [12-14]. They maintain a slow-cycling state to avoid cellular damage from reactive oxygen species (ROS) and to ensure life-long hematopoietic capacity [15,16]. In contrast to these BM-resident quiescent HSPCs, ex vivo manipulated HSPCs are subjected to a variety of stressors, including increases in oxygen tension, fluctuations in various nutrients and growth factors, and accumulation of various metabolites, which can affect their long-term repopulation capacity [17]. In fact, it has been demonstrated that hyperactivation of mammalian target of rapamycin complex 1 (mTORC1) drives HSPCs from quiescence into rapid cycling with increased mitochondrial biogenesis and elevated levels of ROS, resulting in premature exhaustion of HSPCs [18,19]. Likewise, deletion of the 5′ adenosine monophosphate-activated protein kinase (AMPK) regulator liver kinase B1 (LKB1) was found to lead to depletion of HSPC pools and impaired hematopoiesis [20-22]. The main purpose of the present study was to evaluate whether an LL-37 preconditioning strategy is an acceptable clinical application for increasing BM reconstitution with a limited number of HSPCs.

MATERIALS AND METHODS

Animals

CD45.1 and CD45.2 C57BL/6 congenic male mice, maintained in our facility, were used in the experiments at age 8∼12 weeks. The mice were housed in sterilized microisolator cages and received autoclaved food and water ad libitum. All animal experiments were approved by the Animal Care and Use Committee of the Institute of Laboratory Animal Resources, Seoul National University.

BM Nucleated Cells

BM nucleated cells (BMNCs) were prepared by flushing the femurs and tibias of mice. Whole BM cells were suspended in BD Pharm Lyse buffer (BD Biosciences, San Jose, CA) to remove RBCs, washed, and then resuspended with appropriate assay media for further experiments.

Hematopoietic Cell Lines

Human promyelocytic leukemic cell line HL-60 was obtained from the Korea Cell Line Bank and maintained in RPMI 1640 medium supplemented with 10% FBS and antibiotics in humidified atmosphere of 5% CO_2 and 95% air at 37°C.

Transwell Migration Assay

The transwell migration assay was performed as described previously [9]. Unless indicated otherwise, the cells were briefly resuspended in assay media (RPMI 1640 containing 0.5% BSA, 0.5 mM calcium chloride, and 5.5 mM glucose) and equilibrated for 30 minutes at 37°C. An assay medium (650 μL) containing test reagents was added to the lower chambers of a Costar transwell plate (Corning, Tewksbury, MA). Cells were resuspended in assay medium (1×10^7 BMNCs or 2×10^6 HL-60 cells/mL), after which 100 μL of cell suspension was loaded on the upper chambers using 5-μm pore filters, followed by a 3-hour incubation (37°C, 5% CO_2). Migrated cells from the lower chambers were counted using a FACSAria II analyzer (BD Biosciences) to score the migration of cells to the test regents.

For the scoring of clonogenic cell migration, cells harvested from the lower chambers were plated in colony-forming units-macrophage (CFU-M) colony-forming assays. Preconditioned cells were prepared by incubating cells with each compound. These preconditioned cells were loaded onto the upper chamber for the migration assay. In some experiments, preconditioned cells were thoroughly washed before use. CXCL12 and LL-37 were purchased from ProSpec (East Brunswick, NJ) and AnaSpec (Fremont, CA).

CFU-M Colony-forming Assay

Migrated cells were resuspended in human methylcellulose base media provided by the manufacturer (R&D Systems, Minneapolis, MN) supplemented with 10 ng/mL recombinant murine macrophage stimulating factor (ProSpec) for the CFU-M assay. Cultures were incubated for 7 to 15 days, at which time they were scored for the number of CFU-M colonies under an inverted microscope.

Measurement of Cytosolic Free Calcium Changes

HL-60 cells were loaded with Fluo4 NW calcium-indicating dye (Molecular Probes, OR) according to the manufacturer's instructions with some modification. In brief, HL-60 cells were loaded with calcium-indicating dye for 30 minutes in 5% CO_2 at 37°C. After washing, the cells were resuspended with RPMI 1640 medium containing 0.5% BSA (2×10^6 cells/mL) and then loaded at 50 μL/well onto black 96-well plates with opaque bottoms (Corning) with the same volume of LL-37–containing (1000 nM), AMPK activator (AICAR)-containing (4 mM), or control media, followed by incubation for an additional 30 minutes in 5% CO_2 at 37°C. The plates were transferred to a microplate reader (Infinite F200 Pro; Tecan Group, Switzerland). Fluorescence was recorded at 37°C with excitation wavelength of 485 nm and an emission wavelength of 510 nm.

Immunoblotting

HL-60 cells were cultured with RPMI 1640 medium containing 10% FBS and antibiotics in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. In some groups, calcium chloride was supplemented up to 4.5 mM. Protein extracts were separated on a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS/PAGE) gel and then transferred to a nitrocellulose membrane (Hybond ECL; Amersham Life Sciences, Little Chalfont, UK). These extracts were probed with appropriate primary and secondary antibody pairs. Primary antibodies were p-4E-BP1, p-GSK-3β, caspase-3 (#9451, #5558, and #9661, respectively; Cell Signaling Technology, Danvers, MA), and β-actin (#A5441; Sigma-Aldrich, St Louis, MO). The membranes were developed with an ECL reagent (Thermo Scientific, Rockford, IL), dried, and then exposed to film.

Proliferation Assays

For proliferation assays, HL-60 cells were first resuspended in RPMI 1640 medium containing 5% FBS and additional calcium (up to 2.5 mM) in the presence or absence of LL-37. The cell numbers were scored every 24 hours.

Competitive Repopulation Assay

BMNCs were prepared as described above. Isolated BMNCs from CD45.1 congenic C57BL/6 mice were preconditioned with LL-37 (500 nM), lithium chloride (LiCl; 10 mM), AICAR (2 mM), or control media and mixed with normal competitor BMNCs (1×10^6) derived from CD45.2 congenic mice at a 1:1 ratio. Cells were injected through the tail vein of CD45.2 C57BL/6 mice that were lethally irradiated at 1000 cGy approximately 18 hours before transplantation.

Each transplantation group was consisted of 5 recipients. After transplantation, PB samples were obtained to determine donor chimerism. Level of chimerism was determined by the presence of $CD45.1^+$ or $CD45.2^+$ cells. PB samples were obtained from the retroorbital plexus of recipients starting at week 2. Complete blood counts were performed with a VetScan HM2 hematology system (Abaxis, Union City, CA). The PB samples were evaluated by flow cytometry analysis for quantification of $CD45.1^+$ and $CD45.2^+$ cells from the $Gr\text{-}1^+$ population. After the lysis of RBCs, nucleated cell suspensions were evaluated with a FACSAria II, using analyses designed to exclude dead cells, debris, and platelets. Allophycocyanin-conjugated anti-mouse CD45.1 (#17-0453), FITC-conjugated anti-mouse CD45.2 (11-0454), and PE-conjugated anti-mouse Gr-1 (#12-5931) antibodies (all from eBioscience) were used to stain the PB samples.

Statistical Analysis

Data are presented as mean and SEM unless noted otherwise. All differences were analyzed using the Student t test. Statistical analysis for the contribution of transplanted BMNCs in the hematopoietic repopulation was performed using nonparametric methods [23] with the nparLD package in R [24]. A P value $<.05$ was considered statistically significant.

RESULTS

The LL-37 Preconditioning Effect is Specific to Clonogenic Cells

Significantly increased migration of HSPCs in response to CXCL12 in the presence of LL-37 has been reported

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