Rac RhoGTPases Regulate Stem Cell Interaction With Bone Marrow Microenvironment and Are Novel Targets For Stem Cell Mobilization

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Rac members of the Ras-related Rho GTPase family regulate mammalian cell cytoskeleton, survival and proliferation. We have implicated Rac1 in short-term hematopoietic stem/progenitor (HSC/P) cell engraftment (Gu Y, et al., *Science*, 2003) and Rac2 in HSC/P mobilization (Yang FC, et al., *PNAS*, 2001). Indeed, Rac proteins are activated via β_1-integrins, CXCR4 and c-kit, all receptors implicated in homing and mobilization. Recent data examining the function of CXCR4 have been interpreted to show that mobilization and engraftment are mirror image processes. Using both a genetic and a pharmacological approach, we examined the role of Rac proteins in mobilization, engraftment, and steady-state hematopoiesis. Here we demonstrate that whereas Rac1-/- HSC/P fail to engraft after transplantation, deletion of Rac1 by Cre-mediated deletion of floxed Rac1 after engraftment does not significantly affect either blood formation or HSC/P mobilization. However, Rac1-/-;Rac2-/- HSC/P dramatically fail to sustain steady-state hematopoiesis *in vivo* (< 5% contribution by 6 months) leading to a replacement of hematopoiesis by non-gene deleted cells expressing Rac1. Spatial distribution of fluorescently labeled, transplanted Lin-/c-kit+ bone marrow (BM) cells in the endosteal space was defective in Rac1-/- cells (25%) versus wild-type (39%), suggesting defective retention of Rac1-/- HSC in the stem cell niche after engraftment. Rac1-/-;Rac2-/- lin-/c-kit+ cells showed a more severe defect in spatial localization to the endosteum (19% vs. 39% in wild-type). *In vitro*, Rac1-/- HSC/P also showed severely decreased cobblestone area formation ability (> 95% reduction in primitive CAFC frequency) but had normal transendothelial migration. In contrast, Rac2-/- HSC/P demonstrated normal short-term engraftment and only mild defects in these assays. Induction of combined Rac1 and Rac2 deficiency induces a striking mobilization of progenitor cells (Gu Y, et al., *Science*, 2003) while Rac1 re-expression by retrovirus-mediated gene transfer into these mobilized HSC is sufficient to affect engraftment of HSC/P into the BM (9-fold increase in engraftment ability in a competitive repopulation assay). To further exploit the identification of Rac as a regulator of HSC retention in BM, we employed a newly identified small molecule, NSC23776, specifically designed to block the interaction of Rac proteins with activating GTPase exchange factors. Administration of NSC23766 in a single dose of 2.5 mg/kg i.p. induced a doubling of circulating hematopoietic HSC/P in the mobilization-resistant wild-type C57Bl/6 mouse strain. In these mice, the HSC/P mobilization peaked at 6 hours after administration, returning to normal values by 24 hours after drug administration. Larger doses of

NSC23766 did not further increase the mobilization. The peak of mobilization corresponded with decreased levels of phosphorylation in BM of p21-activated kinase-1 (p-PAK1), a known Rac effector, at 6 hours after NSC23766 administration. The inhibition of PAK phosphorylation was reversed at 24 hours, corresponding to the return of HSC/P mobilization to basal levels. To determine if the effect of the inhibitor was associated with specific inhibition of Rac1 versus Rac2, we incubated BM $Lin^-/c\text{-}kit^+$ cells with SDF-1α and NSC23766. At a dose as low as 10 μM, both Rac1 and Rac2 activation were inhibited, in agreement with the proposed mechanism of action of NSC23766. In addition, in Rac2-/- mice, which have increased circulating HSC/P at baseline, administration of NSC23766 at the dose of 1 mg/kg i.p. was sufficient to double the number of circulating CFU-C compared to PBS control as early as 3 hours after administration suggesting that the effect of Rac1 inhibition on the background of Rac2-deficiency is additive *in vivo*. Indeed, increased HSC/P circulation was maintained for a minimum of 24 hours in these mice. Transplantation of NSC23766-mobilized C57Bl/6 peripheral blood HSC/P led to engraftment at levels equivalent to or modestly higher than G-CSF-mobilized peripheral blood. Altogether these data suggest distinct roles for Rac1 versus Rac2 in retention of HSC/P in the BM endosteal space implying that engraftment and mobilization are not mirror image processes. In addition, RacGTPases clearly define critical molecular pathways activated in the stem cell niche and are thus important molecular therapeutic targets.