Int J Hematol (2014) 99:695–698
DOI 10.1007/s12185-014-1588-9

Bone marrow Schwann cells induce hematopoietic stem cell hibernation

Satoshi Yamazaki · Hiromitsu Nakauchi

Received: 4 April 2014 / Accepted: 25 April 2014 / Published online: 10 May 2014

Abstract Hematopoietic stem cells (HSCs) are clonogenic cells capable of both self-renewal and multilineage differentiation. In adult mouse bone marrow (BM), most HSCs remain in the non-dividing G_0-phase of cell cycle, in close contact with supporting cells known as the HSC "niche". In the present study, we focused on signaling mechanisms that regulate stem cell dormancy in the BM niche. We show that TGF-β type II receptor deficiency causes reduced phosphorylation of Smad2/3 and impairs long-term repopulating activity in HSCs, suggesting a significant role for TGF-β/Smad signaling in hematopoiesis. Furthermore, we aimed at defining the candidate BM niche responsible for homeostasis of hematopoiesis, and revealed that non-myelinating Schwann cells sustain HSC hibernation by converting TGF-β from its latent to its active form.

Keywords Hematopoietic stem cell · Hibernation · TGF-β signaling · Schwann cell

Introduction

Hematopoietic stem cells (HSCs) reside in the bone marrow (BM) niche while remaining in a state of quiescence, and are recruited into the cell cycle at long intervals [1–4]. This unique property of HSCs is in some ways reminiscent of hibernation in mammals [5, 6]. Although a number of cytological studies have attempted to characterize a candidate niche, there have been few reports of the inter- and intra-cellular signaling mechanisms that underlie HSC 'hibernation'. This is largely due to the paucity of HSCs, which makes the use of traditional signal transduction assays difficult [7]. To address these issues, we developed a novel assay based on in-droplet single-cell staining and quantitative fluorescence imaging analysis [7, 8]. Using this assay system, we demonstrated that HSCs freshly isolated from the BM lack lipid raft clustering, exhibit repression of the AKT-FOXO signaling pathway [9, 10], and express abundant p57Kip2 cyclin-dependent kinase inhibitor [11, 12]. Subsequent studies also demonstrated that lipid raft clustering induced by cytokines is essential for HSC cell cycle re-entry [13].

Inhibition of lipid raft clustering in contrast caused sustained nuclear accumulation of FOXO transcription factors (FOXO1, FOXO3, FOXO4, and FOXO6) and induced HSC hibernation ex vivo. Among niche signals examined, transforming growth factor-beta (TGF-β) efficiently inhibited lipid raft clustering and induced p57Kip2 expression, leading to HSC hibernation [14]. These data reveal a critical role for lipid rafts in HSC fate determination, and establish the importance of TGF-β in the control of HSC hibernation in the BM niche. Moreover, we demonstrated that TGF-β type II receptor-deficient HSCs show low-level Smad activation and impaired long-term repopulating activity, suggesting a critical role for TGF-β/Smad signaling in HSC maintenance [15, 16]. Considering that TGF-β is produced in a latent form by a variety of cells, we searched for effector cells in BM-expressing signal molecules that activate latent TGF-β. In the present report, we describe how we identified Schwann cells in BM as important for the maintenance of HSC hibernation [17].

S. Yamazaki (✉) · H. Nakauchi
Laboratory of Stem Cell Therapy, Center for Experimental Medicine, The Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan
e-mail: y-sato4@ims.u-tokyo.ac.jp

H. Nakauchi
e-mail: nakauchi@ims.u-tokyo.ac.jp

Impaired HSC function in *Tgfbr2*-deficient mice; Smad2/3 are highly activated in HSCs

To establish the role of TGF-β in the control of HSC hibernation, we generated conventional Tgfbr2 knockout mice [18]. As expected from the significant role of TGF-β in cell growth, inflammation and apoptosis, the mice developed a lethal inflammatory disease (LID) [19]. Similarly, transplantation of the immune cells from *Tgfbr2* knockout mice developed the same disease phenotypes due to an uncontrolled over-reaction of lymphocytes [20]. We therefore generated conditional *Tgfbr2*-deficient (*Mx-1-Cre*: $Tgfbr2^{flox/-}$; $Rag2^{-/-}$) mice, which do not develop LID due to a lack of T and B cells. We then injected polyinosinic–polycytidylic acid (pIpC) intraperitoneally three times every other day to induce *Cre* recombinase activity [21]. Two weeks after pIpC injection, total BM cells were infused into lethally irradiated recipients along with the competitor BM cells. In the current study, peripheral blood (PB) myeloid chimerism was used as the indicator of the repopulation ability of test cells. As a result, chimerism of *Tgfbr2*-deficient BM cells over wild type gradually decreased. These results led us to hypothesize that *Tgfbr2*-deficient HSCs exhibit impaired long-term repopulating activity. To address this possibility, we examined the process of cell cycle or the phosphorylation status of Smad2/3, downstream targets of TGF-β signaling. Our results clearly indicate that the levels of phosphorylated Smad2/3 in $Tgfbr2^{del/-}Rag2^{-/-}$ $CD34^{-}KSL$ HSCs 2 weeks after pIpC injection are significantly lower than those in wild-type ($Tgfbr^{+/+}Rag2^{-/-}$ $CD34^{-}KSL$) HSCs. BrdU incorporation assay also revealed that a significantly higher proportion of $Tgfbr2^{del/-}Rag2^{-/-}$ $CD34^{-}KSL$ HSCs entered the cell cycle within a week compared with $Tgfbr^{+/+}Rag2^{-/-}$ $CD34^{-}KSL$ HSCs. Taken together, these results show that loss of *Tgfbr2* causes reduced phosphorylation of Smad2/3, increased cell cycling, and reduced long-term repopulating activity in HSCs, demonstrating that TGF-β/Smad signaling is active in maintaining the hibernation of BM HSCs.

A specialized cell type produces active TGF-β in BM

Although, TGF-β is produced by various types of blood cells, including HSCs, how it is activated remains obscure. The identification and characterization of cell types that activate TGF-β is thus crucial to the understanding of the physiological role of TGF-β in the BM niche. To address this issue, we conducted studies aimed at defining cell type using two different antibodies: anti-LAP and anti-TGF-β. Anti-LAP antibody was used to detect latent TGF-β while anti-TGF-β antibody was used to detect active TGF-β. In BM sections fixed with dry ice-cold ethanol [22], LAP was abundantly detected in various cells known to produce latent TGF-β, including megakaryocytes. However, active TGF-β expression was highly restricted to a small population of LAP-positive cells with a long, spindle-shaped structure. Notably, integrin-β8 (*Itgb8*) [23, 24] was more specifically expressed in the same population of LAP-positive cells. These findings indicate that anti-TGF-β antibody recognizes a specialized cell type with a long, spindle-shaped structure producing active TGF-β in BM (Fig. 1).

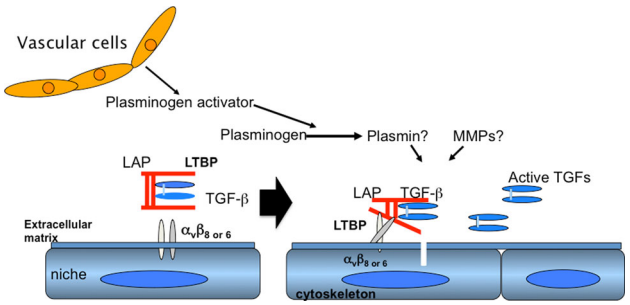

Fig. 1 Processes of Latent TGF-β activation. TGF-βs are secreted as latent complexes containing these forms: active TGF-β, latency-associated protein (LAP), and latent TGF-β binding protein (LTBP). This ternary complex is called the large latent complex (LLC). Because LTBP-1, a member of the LTBP/fibrillin family, covalently binds to extracellular matrix (ECM) proteins, LLC accumulates in the ECM. Under most conditions, TGF-β is secreted as part of the LLC. TGF-β function is therefore largely controlled by activation of latent TGF-β, a process that involves dissociation of bioactive TGF-β from LAP

GFAP-positive cells activate latent TGF-β

We next examined to determine *Itgb8*-positive BM cell type responsible for active TGF-β production. However, RT-PCR analysis showed that neither HSCs nor BM stromal cells express *Itgb8*. We therefore decided to focus on Schwann cells, which are known to express *Itgb8*. There are two types of Schwann cells, myelinating and non-myelinating [25]. Myelinating Schwann cells express myelin basic protein (MBP), but not glial fibrillary acidic protein (GFAP), while non-myelinating Schwann cells express GFAP, but not MBP. We investigated BM sections from *GFAP* promoter-driven GFP transgenic mice [26] and found that most GFP-positive cells stained for active TGF-β. These cells also expressed *Itgb8*, a marker antigen for glial cells. Interestingly, these GFAP-positive cells were present in parallel with neurofilament (NF)-positive axons. Analysis of transverse sections together with immunostaining revealed that GFAP-positive cells are positive for active TGF-β and *Itgb8*, and ensheath NF- and tyrosine hydroxylase (TH)-positive axons.

Furthermore, VE-cadherin positive endothelial cells and GFAP- and *Itgb8*-positive cells were found to be discrete cell populations, located in parallel and in close proximity to each other. These results led us to suggest that non-myelinating Schwann cells in BM are responsible for HSC hibernation.

HSCs exist in contact with GFAP-positive cells in BM

To ask whether HSCs stay in close contact with BM Schwann cells, we performed ArrayScan analysis of BM sections to determine the positional relationship between HSCs and BM GFAP-positive cells. GFAP-expressing Schwann cells were in direct contact with about 20 % of $CD150^{+}CD48^{-}CD41^{-}Lin^{-}$ HSCs [27], whereas 13 and 30 % of HSCs were in contact with osteoblastic cells and vascular cells, respectively. However, 32 % of HSCs stayed apart from any of these cells, perhaps in contact with unidentified cells or may be due to scanning failure. Of particular interest was that Schwann cells prepared from sciatic nerves expressed major HSC niche factor genes such as *Cxcl12* [28, 29], *kitl* [30], *Angpt1* [3], and *Tpo* [31] as well as TGF-β and *Itgb8*. Collectively, these data suggest that BM Schwann cells are prime candidates for constituents of the BM niche.

Denervation of sympathetic nerve results in loss of HSCs

To address the functional significance of BM Schwann cells as a BM niche candidate affecting HSC hibernation, we explored to delete ensheathing GFAP-positive cells in BM via Wallerian degeneration by transecting postganglionic sympathetic nerves [32]. Nerve injury induces a reduction of neuregulin-1 signaling and eventually triggers apoptosis of Schwann cells [33]. For this purpose, the lumbar sympathetic trunk was cut unilaterally at L2, L3, and L4 to denervate sympathetic nerves to the biceps femoris muscle and BM [34]. This surgical strategy revealed a statistically significant decrease in the number of GFAP- and activation of TGF-β-positive cells when compared with sham-operated controls. The number of HSCs on the denervated side continued to decline till day 3, and eventually fell to one-fifth of those in the sham side by day 7, whereas other niches components remained intact even after 1 week following denervation. These findings provide intriguing evidence that loss of Schwann cells after denervation surgery leads to a decrease of HSCs.

Sympathetic nerve denervation compromises dormancy of HSCs

To further confirm loss of HSCs after denervation, competitive repopulation analysis was performed using BM cells harvested 1 month after sympathetic nerve transection. Three months later, we analyzed donor chimerism in PB and observed significantly lower chimerism in mice transplanted with BM cells derived from the denervated side than those from the sham-operated contralateral side. Competitive repopulation assay revealed no significant difference between $CD34^{-}KSL$ cells purified from denervated BM and those from control BM. To explain why reconstitution activity of HSC is decreased after denervation, we examined phosphorylation levels of Smad2/3 in HSCs. The results showed profound down-regulation of pSmad2/3 in HSCs following denervation. Furthermore, cell cycle analysis revealed a significant proportion of HSCs isolated from denervated BM are positive for BrdU, indicating accelerated cell cycle progression. Notably, infusion of active TGF-β suppressed active cycling of HSCs in denervated mice, but latent TGF-β did not. These results support the idea that glial cells are component of BM niche and sustain HSC hibernation by converting latent TGF-β into active form (Fig. 2).

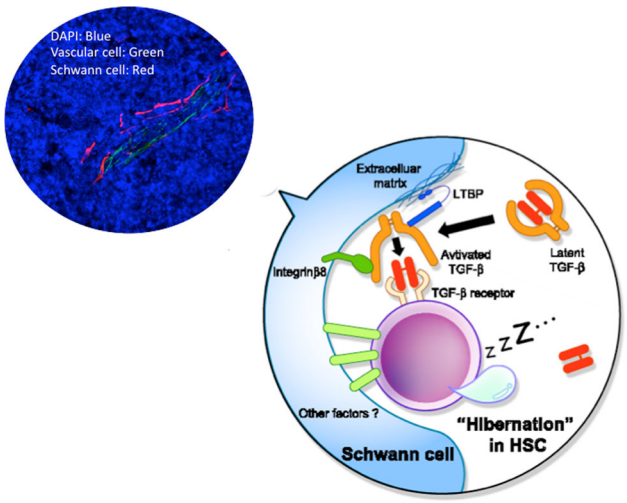

Fig. 2 BM Schwann cells maintain HSC hibernation in the niche. By regulating the activation process of TGF-β, these Schwann cells appear to control hibernation of HSCs in BM. How components of the BM niche are related in the maintenance of homeostasis of HSCs under the influence of the autonomic nervous system remains an intriguing issue. Identification of Schwann cells as BM HSC niche constituents opens a new area of research that links the neural and hematopoietic systems

Conclusion

In present study, we sought to define cells and signals that regulate HSC hibernation in the BM niche. The results of experiments using conditional knockout mouse models revealed the crucial cell type for HSC hibernation to be non-myelinating Schwann cells. These cells produce active

TGF-β, which phosphorylates Smad2/3, leading to cell cycle progression. We propose that these cells are components of BM niche and sustain hibernating HSCs by converting latent TGF-β into its active form.

Acknowledgments We would like to thank Dr. Masataka Kasai for discussion and critical reading of manuscript.

Conflict of interest The authors declare no conflicts of interest.

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