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Original Article

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Overexpression of heparanase in mice promoted megakaryopoiesis

Ying-Xia Tan[2,3,†], Hao Cui[2,4,†], Lu-Ming Wan[3,†], Feng Gong[3], Xiao Zhang[5], Israel Vlodavsky[6], and Jin-Ping Li[2,1]

[2]Department of Medical Biochemistry and Microbiology, SciLifeLab Uppsala, The Biomedical Center, University of Uppsala, Husargatan 3, 75123 Uppsala, Sweden, [3]Department of Tissue Engineering, Beijing Institute of Transfusion Medicine, No 27, Taiping Road, 100850 Beijing, China, [4]College of Life Science, Jiangxi Normal University, No 99, Ziyang Road, 330022 Nanchang, China, [5]Department of Neuroscience and Pharmacology, University of Uppsala, Box 593, 75124 Uppsala, Sweden, and [6]Cancer and Vascular Biology Research Center Rappaport, Faculty of Medicine, Technion, Box 9649, 31096 Haifa, Israel

[1]To whom correspondence should be addressed: Tel: 0046184714241; e-mail: jin-ping.li@imbim.uu.se

[†]These authors have contributed equally to the work.

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Abstract

Heparanase, an endo-glucuronidase that specifically cleaves heparan sulfate (HS), is upregulated in several pathological conditions. In this study, we aimed to find a correlation of heparanase expression and platelets production. In the transgenic mice overexpressing human heparanase (Hpa-tg), hematological analysis of blood samples revealed a significantly higher number of platelets in comparison with wild-type (Ctr) mice, while no significant difference was found in leukocytes and red blood cell number between the two groups. Total number of thiazole orange positive platelets was increased in Hpa-tg vs. Ctr blood, reflecting a higher rate of platelets production. Concomitantly, megakaryocytes from Hpa-tg mice produced more and shorter HS fragments that were shed into the medium. Further, thrombopoietin (TPO) level was elevated in the liver and plasma of Hpa-tg mice. Together, the data indicate that heparanase expression promoted megakaryopoiesis, which may be through upregulated expression of TPO and direct effect of released HS fragments expressed in the megakaryocytes.

Key words: heparan sulfate, heparanase, megakaryopoiesis, platelets

Introduction

Megakaryopoiesis is a process of hematopoietic stem cells (HSC) differentiation to megakaryocytes that produce platelets. HSC in the liver, kidney, spleen and bone marrow are capable of producing different blood cells depending on the signals they receive (Dexter et al. 1979). The primary cytokine for HSC differentiation to megakaryocytes is thrombopoietin (TPO) (Kuter 2014). Upon binding of TPO to myeloproliferative leukemia protein (c-MPL), Janus kinase (JAK) becomes activated and phosphorylates c-MPL, which further activates STAT3/5, MAPK and PI3K pathways (Nivarthi et al. 2016). Apart from TPO, stromal cells and molecules in the extracellular matrix (ECM) also play roles in the process of megakaryopoiesis (Levine et al. 1985; Roberts et al. 1988).

Heparan sulfate proteoglycan (HSPG) is a macromolecular glycoconjugate ubiquitously expressed on the cell surface and in the ECM. Heparan sulfate (HS) side chains, belonging to the family of glycosaminoglycans (GAGs), are essential in animal development and play critical roles in homeostasis as well as under various disease conditions. The diverse effects of HS are primarily attributed to its interaction with a multitude of proteins, including growth factors and their receptors, cytokines, chemokines, enzymes and ECM proteins (Li and Kusche-Gullberg 2016). Several studies reported that

GAGs (HS, heparin, dermatan sulfate, chondroitin sulfate, hyaluronic acid) play a role in the process of megakaryopoiesis (Kashiwakura et al. 2006; Maurer and Gezer 2013).

Heparanase is an endo-glucuronidase that specifically cleaves HS polysaccharide chains. Heparanase enzymatic activity was initially detected in human platelets (Wasteson et al. 1976), owing to the relatively high expression level of the enzyme in these cells (Freeman and Parish 1998; Cui et al. 2016). Notablyheparanase is elevated in a number of human pathologies, e.g., cancer, inflammatory disorders, diabetes, kidney dysfunction (Vlodavsky et al. 2012; Sanderson et al. 2016) and its activity is implicated in neovascularization, inflammation and autoimmunity (Vlodavsky et al. 1992, 2008). Heparanase modulates ECM and basement membrane structures through degradation of HS, leading to release of the immobilized biological molecules, e.g., growth factors and cytokines (Vlodavsky et al. 1992). Overexpression of heparanase in mice results in extensive degradation of HS accompanied with diverse phenotypes that are plausibly related to alterations in HS structure (Escobar Galvis et al. 2007; Zhang et al. 2012).

In this work, we investigated the implications of heparanase in megakaryopoiesis. Results obtained from in vivo and in vitro experiments revealed a novel activity of heparanase in platelet production, promoting HSC differentiation to megakaryocytes. Our data suggest that this biological activity of heparanase is accomplished through at least two mechanisms, stimulation of TPO expression in the liver and degradation of HS to enhance MK maturation. The results offer a plausible explanation for the high level of heparanase in platelets. This function of heparanase likely has important clinical implications to be identified, e.g., in patients with hematological and neoplastic diseases where heparanase expression is often upregulated.

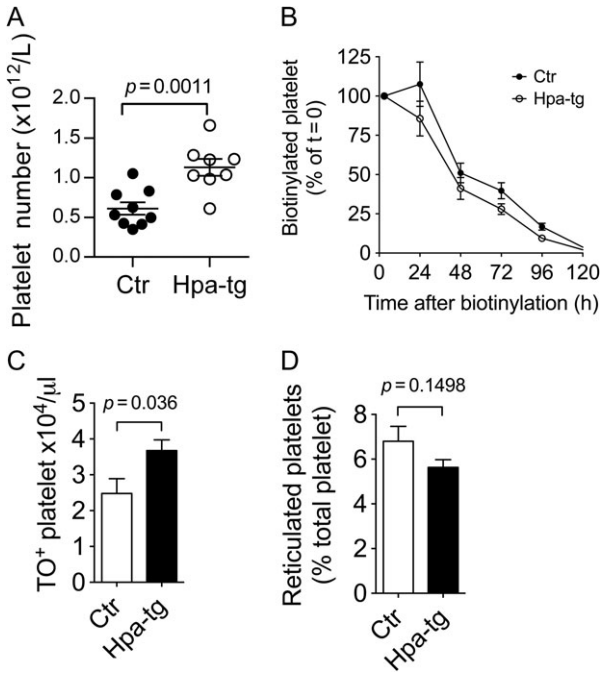

Fig. 1. Analysis of platelets in blood. (**A**) Hematological analysis of platelets in the blood of Hpa-tg and Ctr mice (Hpa-tg $n = 8$; Ctr $n = 9$). (**B**) FACS analysis of in vivo biotin-NHS labeled platelets from tail blood over 5 days ($n = 4$ in each group). (**C**) FACS analysis of thiazole orange (TO) stained platelets in blood collected from Hpa-tg and Ctr mice. (**D**) Percentage of TO positive (reticulated) cells of total CD61 positive platelets (mean ± SE of eight mice in each group for the data in C, D) analyzed by FACS.

Results

Increased platelets production in mice overexpressing heparanase

Blood was collected by heart punctuation from age and sex matched cohorts of heparanase transgenic (Hpa-tg) and control (Ctr) mice into EDTA-tubes. Hematological analysis was performed at the Clinical Pathology Laboratory, University Animal Hospital, Sweden. The results revealed a significantly higher number of platelets in Hpa-tg than in Ctr mice (Figure 1A), while the total number of leukocytes was slightly higher (Supplementary Figure S1A) and the number of red blood cells (RBC) was slightly lower (Supplementary Figure S1B) in Hpa-tg mice, suggesting that the increased platelet number is not a consequence of an overall hyper-hematopoietic activity in the Hpa-tg mice. Monitoring of in vivo biotin-labeled platelets showed a similar clearance rate of aged platelets in Hpa-tg and Ctr mice (Figure 1B), indicating that the higher platelet counts in Hpa-tg blood is not a result of delayed/impaired clearance. Examination of reticulated platelets after Thiazole orange (TO) staining revealed an increased total number of TO positive platelets in Hpa-tg vs. Ctr blood (Figure 1C), reflecting a higher rate of platelet production. However, the ratio of reticulated/total platelets in Hpa-tg blood was essentially comparable to that of Ctr mice (Figure 1D), demonstrating that the platelets in Hpa-tg mice utilize a normal maturation process. Thus, it can be established that more matured platelets were produced in Hpa-tg vs. Ctr mice.

More megakaryocyte cells in the tissues of Hpa-tg mice

To find out the mechanisms behind the increased number of platelets in Hpa-tg mice, we first examined megakaryocytes (MK) activity. Analysis of total CD41 (GPIIb/IIIa also known as integrin α2b and GPIIb) positive cells collected from bone marrow revealed no significant difference in MK ploidy distribution (Supplementary Figure S2). However, staining of bone marrow (BM), spleen and lung sections identified more CD61 (α5β3, vitronectin receptor) positive MK cells in the tissues of Hpa-tg than in the corresponding Ctr tissues (Figure 2A). Counting the CD61 positive cells in the tissue sections resolved a significant higher total number of MK in these tissues of Hpa-tg mice (Figure 2B). This finding was further verified by in vitro differentiation of MK from cultured primary BM cells. FACS analysis confirmed significantly higher number of CD61 positive MK cells in the Hpa-tg BM cultured for 4 days (Figure 3A). However, there was no difference in proplatelet formation between the CD61 positive MK isolated from Hpa-tg and Ctr mice (Figure 3B and C). The data suggest that the higher number of platelets in Hpa-tg mice is a result of increased number/percentage of MK cells.

Upregulated TPO production in the liver of Hpa-tg mice

Obviously, the Hpa-tg mice exhibited a higher potency of megarkayocytes differentiation, leading to increased production of platelets. As thrombopoietin (TPO) is the major cytokine stimulating hematopoietic cells differentiation to MK, we investigated whether heparanase expression affected TPO production. Western blot analysis of liver tissue lysates detected several positive bands, ranging from 130–22 kDa, where the signal of the 50 kDa band is substantially higher in all four Hpa-tg samples; while the signals of 60 kDa and 22 kDa are slightly stronger in the Hpa-tg liver (Figure 4A). Because the molecular mass of TPO varies due to proteolytic

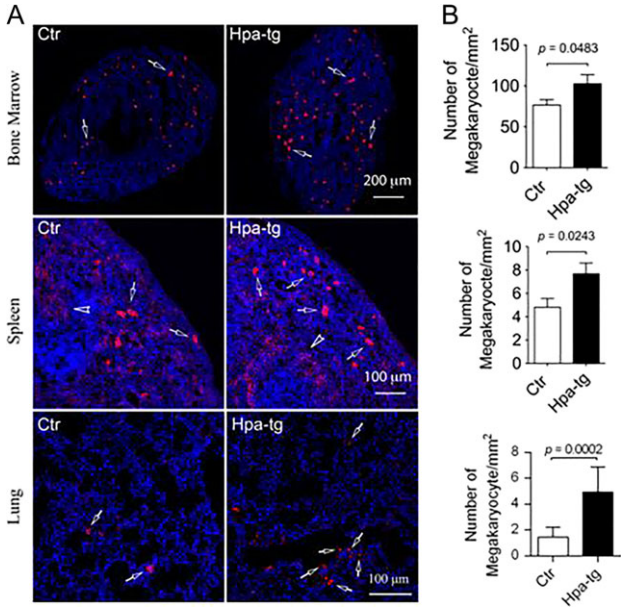

Fig. 2. Detection of megakaryocytes (MK) in mouse tissues. (**A**) Cryosections of bone marrow (after decalcification), spleen and lung from Hpa-tg and Ctr mice were stained with anti-CD61 antibody. The positive cells (MK; *red*) were counted in each section ($n = 4$ mice in each group) under a microscope and the results are presented in (**B**). Blue shows DAPI staining.

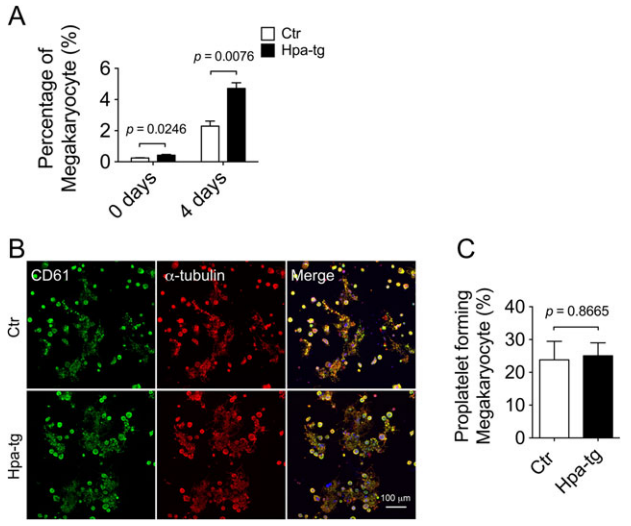

Fig. 3. Analysis of bone marrow-derived megakaryocytes (MK). Bone marrow cells from Hpa-tg and Ctr mice were cultured in the presence of TPO. (**A**) Percentage of proplatelet forming MK was quantified by counting alpha tubulin positive tabula structures extended from MK cells. The data are from five microscopic fields of each slide ($n = 4$ mice in each group). (**B**) Immunostaining of the cells with anti-CD61 (*green*) and anti-alpha tubulin (*red*) antibodies after culturing for 4 days. (**C**) FACS analysis of in vitro cultured bone marrow cells. Percentage of MK is calculated by counting CD61 positive cells in a total of 200,000 cell events ($n = 4$ mice in each group).

cleavage and degradation of the attached glycans (Kato et al. 1998), the appearance of multiple bands in the Western blot makes it difficult to assess the amount. Instead, we used an ELISA assay (R&D Systems), which confirms the higher concentration of total TPO in the Hpa-tg liver samples (Figure 4B). The elevated expression of TPO was

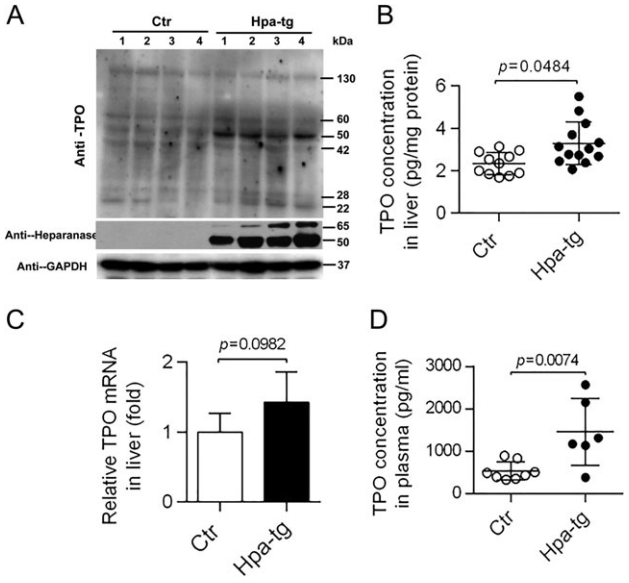

Fig. 4. Analysis of TPO levels. (**A**) Total protein of liver lysates (80 μg) was analyzed by Western blotting using anti-TPO and anti-GAPDH antibodies. (**B**) ELISA analysis of TPO in the liver lysates of 100 μL (40 μg/μL) (Ctr $n = 11$; Hpa-tg $n = 13$). The standard curve was generated from mouse TPO standard dilutions range of 0–4000 pg/mL. (**C**) Thrombopoietin expression in the liver analyzed by QPCR ($n = 5$ in each group). (**D**) ELISA analysis of TPO in the plasma (Ctr $n = 8$; Hpa-tg $n = 6$).

further confirmed by QPCR analysis (Figure 4C). Additional analysis of plasma also found higher level of TPO in the Hpa-tg samples (Figure 4D), implying a direct effect of TPO in the platelets producing organs.

Upregulated heparanase expression in maturing MK cells

To find out the effect of heparanase expression in MK cells, we cultured fetal liver cells from both Hpa-tg and Ctr mice in the presence of TPO. Maturation of MK was confirmed by formation of proplateletes forming MK (Supplementary Figure S3). Immunostaining confirmed overexpression of heparanase in the Hpa-tg MK, partially overlapping with CD62P (Figure 5; lower panels). Notably, substantial expression of heparanase was also detected in platelets releasing Ctr MK, but not in non-matured Ctr MK (Figure 5; upper panels; *arrow*), indicating an association of heparanase expression and platelet production.

Heparanase overexpression leads to production of shorter HS chains in MK cells

We have previously reported that heparanase expression resulted in increased production of HS fragments in the liver (Escobar Galvis et al. 2007). This effect of heparanase was examined in MK matured from a primary culture of fetal liver hematopoietic cells. For analysis of HS structure, matured MK cells after culturing for 4 days (Supplementary Figure S3) were cultured in the presence of $Na_2{}^{35}SO_4$ to metabolically label HS. The cells and medium fractions were then collected for isolation of sulfated GAGs. Quantification of ^{35}S in the purified GAGs prior and after chondroitinase ABC digestion revealed that GAGs from Ctr MK cell fraction consisted of approximately 80% chondroitin sulfate (CS)

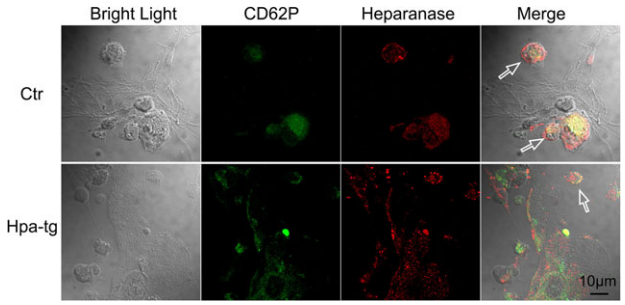

Fig. 5. Heparanase expression in fetal liver-derived megakaryocytes (MK). Fetal liver cells from Hpa-tg and Ctr mice were collected and cultured in the presence of TPO. Immunohistochemical staining of the in vitro matured MK cells after culturing for 4 days show expression of heparanase and CD62P co-localized in both platelet-releasing MK and released platelets. Upregulated heparanase expression was also observed in the platelet-releasing Ctr MK cells (*arrows*).

and 20% HS, while Hpa-tg MK had slightly higher (about 23%) proportion of HS. Remarkably, medium conditioned by Hpa-tg MK contained almost 5-fold more HS than that of Ctr MK, indicating an intensive shedding of HS chains from Hpa-tg MK. Indeed, analysis of purified HS by size exclusion chromatography confirmed extensive fragmentation of HS in the Hpa-tg MK medium (Figure 6A) and cell (Figure 6B) fractions.

It has been shown that addition of sulfated polysaccharides, e.g., HS, stimulated megakaryopoiesis of cultured blood cells from human umbilical cord (Maurer and Gezer 2013) and peripheral blood (Kashiwakura et al. 2006), suggesting stimulatory effect of HS in platelet production. In line with this, we hypothesized that the HS fragments accumulated in the culture media of Hpa-tg MK may have stimulated megakaryopoiesis as the exogenously added HS. To test this, we cultured bone marrow cells in the presence of TPO and low molecular weight heparin (LMWH). Analysis of CD41 expression found a dose-dependent effect of LMWH in stimulation of MK maturation (Supplementary Figure S4).

Discussion

Heparanase, the sole HS degrading endo-glucuronidase in mammals is expressed at very low levels in majority of tissues under healthy conditions, with the exception of human placenta and platelets. The robust expression of heparanase in human platelets led to discovery of the enzyme (Wasteson et al. 1976) and later on purification of the protein and cloning of the heparanase gene from these cells (Hulett et al. 1999). However, the reason why human platelets contain such a high level of heparanase remains unknown. Our previous study demonstrated that heparanase expression in platelets is related to its activities (Cui et al. 2016). In this study, we focused on the physiological implications of heparanase in megakaryopoiesis and platelets production.

Hematological analysis revealed significantly more platelets in the blood of transgenic mice overexpressing human heparanase (Hpa-tg) than that of wild-type mice (Ctr) (Figure 1A).

The comparable decline in the amount of biotinylated platelets in blood circulation between Hpa-tg and Ctr mice excludes a defect in platelet clearance in Hpa-tg mice. An increased production of platelets in the Hpa-tg mice is therefore conceivable. However, this increase apparently is not a result of hyper-proliferation of MK cells, as the number of proplatelet forming MK in the population of cultured bone marrow cells did not differ between MK from Hpa-tg and Ctr mice

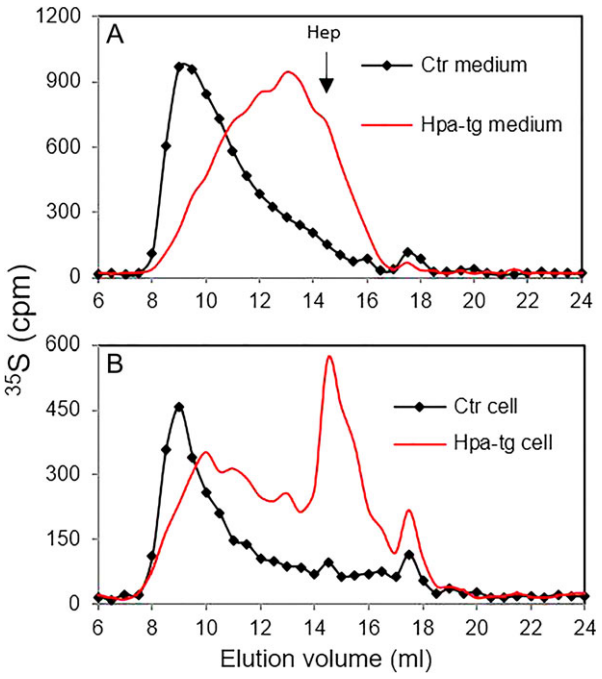

Fig. 6. Analysis of HS molecular structure. Metabolically ^{35}S-labeled heparan sulfate (HS) isolated from in vitro matured megakaryocytes was analyzed on Superose-12 gel filtration column. Effluent fractions were assayed for radioactivity. The *hep-arrow* in the upper panel indicates the elution volume of heparin (15 kDa). The *lines* show that heparanase overexpressing MK produced substantially shortened HS fragments in both (**A**) medium and (**B**) cellular fractions.

(Figure 3C). This is also evidenced by the similar proliferation rate of MK derived from the two groups of mice (Supplementary Figure S2). Nonetheless, the number of MK residing in the organs that produce platelets in adulthood, e.g., spleen, bone marrow and lung of the Hpa-tg mice was higher than that found in the Ctr mice (Figure 2). Thus, the increased platelet number in the circulation of Hpa-tg mice is most likely a result of increased MK cells differentiation of hematopoietic stem cells (HSC) boosted by overexpressed heparanase. This hematopoiesis seems "selective" towards to megakaryopoiesis lineage as the numbers of leukocytes and red blood cells were essentially the same. The hyper-megakaryopoiesis in Hpa-tg mice is, at least partly, promoted by the increased level of TPO known to stimulate hematopoietic cell differentiation to MK. Our finding resembles the recently proposed model, where a megakaryocyte-biased HSC directly differentiates into megakaryocyte progenitors, bypassing the classical intermediate commitment stages (Woolthuis and Park 2016). This model plausibly explains the differential effect of heparanase expression on the number of platelets vs. leukocytes and red blood cells in the Hpa-tg mice.

Moreover, our earlier study revealed that overexpression of heparanase resulted in a substantial increase of highly sulfated (heparin-like) HS fragments in the liver, and the heparin-like fragments showed a higher potency in simulation of FGF activities (Escobar Galvis et al. 2007). Thus, the upregulated TPO expression in Hpa-tg liver may also be ascribed to an effect of increased growth factor activity that stimulates TPO expression (Sungaran et al. 2000). Recent study shows that TPO production in the liver is regulated via JAK2-STAT3 signaling (Grozovsky et al. 2015); while heparanase expression has been found to induce STAT3 phosphorylation (Cohen-Kaplan et al. 2012). By all means, the non-enzymatic activity of heparanase in stimulation of cellular

signaling pathways may also contribute to a hyper activity of liver cells (Riaz et al. 2013).

It has been reported that GAGs, including HS, exert positive regulatory effects on megakaryopoiesis (Han et al. 1996; Kashiwakura et al. 2006). Treatment with low molecular weight heparin (LMWH) promoted megakaryopoiesis in mice through several potential mechanisms, including neutralization of the inhibitory effect of platelet factor 4 (PF4) (Shen et al. 1994), as heparin (or negatively charged GAG molecules) has a high affinity towards PF4 (Maccarana and Lindahl 1993; Krauel et al. 2012). Thus it is possible that the excess HS fragments generated by heparanase action in Hpa-tg mice contributes to the hyper-megakaryopoiesis through at least two distinct, yet associated mechanisms. Apart from the effect on TPO expression, the highly sulfated HS fragments produced in the liver (Escobar Galvis et al. 2007) and in the MK cells (Figure 6) neutralize PF4 that was found inhibitory to TPO expression in bone marrow cells (Sungaran et al. 2000).

The most important finding of this study is the correlation of heparanase expression and MK differentiation/platelets production. These results strongly argue for the physiological function of heparanase in platelets production. In light of these findings, it is of interest to examine whether the heparanase-associated megakaryopoiesis has any clinical relevance in patients with thrombocyte-related hematological diseases. Further, given that heparanase also degrades heparin (Gong et al. 2003), it is of worth to investigate whether increased expression of heparanase, e.g., in cancer patients, have any impact on the activity of exogenously delivered heparin. This brings up an additional clinically relevant question, i.e., whether heparin-induced thrombocytopenia (HIT) is associated with heparanase expression? Thus, it would be of importance to assess heparanase expression levels in patients suffering from HIT.

In summary, our results propose an important novel function of heparanase for megakaryopoiesis and platelets production. Since platelets play pivotal roles in hemostasis, tumor metastasis and inflammation, a rational hypothesis is that heparanase inhibitors could potentially fortify the effects of anti-platelet drugs, e.g., aspirin. Several heparanase inhibitors are currently under development and clinical testing in cancer patients (McKenzie 2007; Rivara et al. 2016). Application of such inhibitors may have a dual effect on cancer patients who are complicated with micro-thrombotic symptoms (Vlodavsky et al. 2016). Heparanase inhibitors are also expected to inhibit platelet-tumor cell aggregation and hence diminish the protective effect of platelets on circulating tumor cells (Leblanc and Peyruchaud 2016). It should be of interest to find out whether this function of heparanase is associated with the clinically observed hyper-thrombotic phenomena in cancer patients as elevated levels of heparanase are detected in the majority of cancer patients.

Materials and methods

Animals

Transgenic mice overexpressing heparanase (Hpa-tg) were generated as described (Zcharia et al. 2004). The mice were backcrossed with C57bl mice for more than 10 generations and wild-type C57bl mice were used as control (Ctr). Mice were maintained at the animal facility, Biomedical Center, Uppsala University. The local ethic committee approved the procedures involving animal experiments, and the experiments were conducted in accordance with animal welfare regulations. The low molecular weight heparin (Dalteparin) was purchased from drug store in China.

Reagents

Biotin N-Hydroxysuccinimide ester (biotin-NHS) (H1759) was from Sigma-Aldrich. PE-conjugated streptavidin (#12-4317) was obtained from eBioscience. Rabbit anti-CD61 antibody was purchased from Abcam. Rabbit anti-heparanase antibodies #1453 were previously described (Zetser et al. 2004). Rabbit anti-GAPDH, anti-CD62P and secondary HRP-conjugated anti-mouse and rabbit IgG were obtained from Santa Cruz. Secondary Alexa fluor 488 or 594 conjugated goat anti-mouse or rabbit antibodies were from Invitrogen. PE-conjugated hamster anti-mouse and CD61 (platelet identification marker), FITC-conjugated hamster anti-mouse CD61 and CD41, FITC-conjugated rat anti-mouse CD62P (P-selectin) and isotype IgGs were from BD Pharmingen. Parengy's decalcification solution was from Histolab Products AB (Gothenburg, Sweden). ECL reagent (SuperSignal® West Femto Maximu Sensitivity Substrate) was from Thermo Scientific. TPO, mouse TPO antibody (monoclonal Rat IgG) and Quntikine® ELISA kit for mouse thrombopoietin immunoassay (MTP00) were from R&D Systems. PrimeScript™ RT Master Mix and SYBR® Premix Ex Taq™ II were from Takara Bio, Shiga, Japan.

Collection of blood and hematological analysis

Mouse blood was collected from anesthetized animals by cardiac puncture. The hematological analysis of whole EDTA-treated blood from mice was performed using an automatic hematology analyzer (Advia 2120; Siemens Healthcare, Germany) with veterinary software and settings for mouse blood. The samples from Ctr and Hpa-tg mice were analyzed in parallel. All blood cells dot plots from the instrument were evaluated by an experienced biomedical technologist (Clinical Pathology Laboratory, University Animal Hospital at the Swedish University of Agricultural Sciences, Uppsala, Sweden).

Platelet analysis

Biotin *N*-Hydroxysuccinimide ester (biotin-NHS) (H1759) was dissolved in DMSO (40 mg/mL) and diluted 1:10 with sterile saline immediately before injection. Following tail vein injection of 600 µg biotin-NHS, the tail blood was taken for analysis of biotinylated platelets over 5 days. Whole tail blood (2 µL) was incubated with FITC-conjugated anti-CD61 antibody and PE-conjugated streptavidin (#12-4317) and platelets were identified based on forward and side scatter and on anti-CD61 antibody staining. Total of 200,000 events in each sample were analyzed by flow cytometry.

For analysis of reticulated platelets, whole tail blood (2 µL) was suspended in 100 µL PBS containing 2% fetal bovine serum (FBS). The cell suspension was then incubated with PE-labeled anti-CD61 antibody for 30 min at room temperature. Then, the cells were mixed with 900 µL of TO solution (10 ng/mL), and incubated for 15 min in dark at room temperature. Both CD61 and TO positive platelets were quantified by flow cytometry.

Megakaryocyte ploidy

Bone marrow cells were isolated from femurs as previously described (Heazlewood et al. 2013). Briefly, cells were fixed with cold 70% ethanol at 4°C overnight and washed twice with PBS. The cells were then incubated with anti-CD41 antibody for 45 min and resuspended in 1 mL PBS containing 0.1% Triton X-100, 50 µg/mL

RNAse A and 100 μg/mL propidium iodide (PI). After incubation for 2 h, the cells were analyzed by BD LSR II flow cytometry (BD Biosciences). $CD41^+$ cells were regarded as MK and analyzed for ploidy using FlowJo.

Immunohistostaining

Mice were euthanized by CO_2 and perfused with PBS. Spleen, lung and femurs were dissected and fixed in 4% PFA overnight at 4°C. The femurs were then decalcified in Parengy's decalcification solution for 24 h at room temperature. The tissues were transferred to sucrose solutions (15% and 30%), and then frozen in O.C.T. Cryosections of 10 μm were prepared and stained with rabbit anti-mouse CD61 antibody and secondary Alexa fluor 594 conjugated goat anti-rabbit antibody. MK number in the entire section was counted and normalized to the total tissue area using Image J software.

Cell culture

Primary culture of megakaryocytes (MK) was prepared from fetal liver and bone marrow essentially as described (Shivdasani and Schulze 2005). Briefly, mouse fetal livers were collected from embryos at E13.5. Single-cell suspensions (about 2–4 × 10^7 cells/mL) were prepared by successive passages through 18-, 20- and 22-gauge needles. The bone marrow cells were collected by flush of the bone lumen of adult mice with 0.5 mL sterile PBS. The cells were cultured in DMEM supplemented with 10% FBS, 50 U/mL penicillin, 50 μg/mL streptomycin and 50–75 ng/mL TPO in 10-cm dishes. After 3.5–4 days, mature MK were analyzed as described in the respective figure legends.

Western blot and ELISA analysis

The frozen tissues were homogenized in 1:10 (w/v) CelLytic™ MT Cell Lysis Reagent with protease inhibitor cocktail (Sigma-Aldrich) followed by centrifugation. The lysates were subjected to SDS-PAGE (12%) followed by electro-transfer to PVDF membranes and subsequent blocking in 5% nonfat dry milk dissolved in Tris-buffered saline containing 0.05% Tween. The membranes were probed with primary antibodies against heparanase, mouse TPO and GAPDH, followed by incubation with corresponding secondary antibodies. Signals were developed by using ECL reagent (SuperSignal® West Femto Maximu Sensitivity Substrate) and exposed to film.

For ELISA analysis, the liver lysates were adjusted to 40 μg/μL protein and 100 μL was analyzed using the Quntikine® ELISA kit for mouse thrombopoietin immunoassay. EDTA-plasma of 25 μL diluted with 25 μL of buffer was used for the ELISA analysis

Quantification of gene expression

Total RNA was isolated from frozen liver or cultured bone marrow cells with Trizol (Sigma-Aldrich). Then, 1 μg of RNA was reversely transcribed using PrimeScript™ RT Master Mix (Takara Bio, Shiga, Japan). Quantitative RT-PCR was performed in triplicate using SYBR® Premix Ex Taq™ II (Takara Bio, Shiga, Japan). The primers used for mouse thrombopoietin: sense, 5′-GGCCATGCTTCTTGC AGTG-3′; antisense, 5′-AGTCGGCTGTGAAGGAGGT-3′; mouse CD41: sense, 5′-CTTGAACCTGGACTCTGAGAAG; antisense, 5′-TCCTCTTGACTTGCGTTTAGG; mouse GAPDH: sense, 5′-AG TGGCAAAGTGGAGATT-3′; antisense, 5′-GTGGAGTCATACTGG AACA-3′.

Isolation and analysis of metabolically labeled HS from cells

MK matured in vitro were cultured to 95% confluence and 100 μCi/mL of $Na^{35}SO_4$ (specific activity 1500 Ci/mmol, Perkin Elmer) were added to the culture for 24 h before harvesting. Proteoglycans were purified from cell lysates and media as described (Escobar Galvis et al. 2007). The purified total proteoglycans was further treated with chondroitinase ABC (25mU, Seikagaku) and benzonase (60U, Merck) and re-applied to a 0.5-mL DEAE-Sephacel column to eliminate degraded chondroitin sulfate (CS) and oligonucleotides. HS chains were released from the proteoglycan by alkali treatment (incubation of the samples in 0.5 M NaOH). The chain length of purified HS (5000–10,000 cpm) was analyzed on a Superose-12 column connected to HPLC-system.

Statistical analysis

Two-tailed unpaired Student's *t*-test was used to determine the significance between population means. The data are presented as mean ± SEM.

Key points

- Heparanase expression increased TPO production in liver.
- Heparanase degradation of heparan sulfate modulated megakaryopoiesis in mice.

Supplementary data

Supplementary data is available at *Glycobiology* online.

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Conflict of interest statement

None declared.

Authorship

Tan YX, Cui H, Li JP designed the experiments and analyzed the data. Tan YX, Cui H, Wan LM, Gong F, Zhang X performed experiments. Vlodavsky I and Li JP analyzed the data and wrote the MS.

Abbreviations

BM, bone marrow; biotin-NHS, biotin N-Hydroxysuccinimide ester; CS, chondroitin sulfate; Ctr, control; ECM, extracellular matrix; FBS, fetal bovine serum; GAGs, glycosaminoglycans; HIT, thrombocytopenia; Hpa-tg, transgenic mice overexpressing heparanase; HSC, hematopoietic stem cell; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; JAK, Janus kinase; LMWH, low molecular weight heparin; MK, megakaryocytes; PI, propidium iodide; RBCs, red blood cells; TO, thiazole-orange; TPO, thrombopoietin.

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