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Hematopoiesis in mice is extremely resilient to wide variation in TIMP/MMP balance

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Abstract

Tissue inhibitor of matrix metalloproteinases (TIMPs) are natural inhibitors of matrix metalloproteinases (MMPs) and are associated with normal and pathologic extracellular matrix turnover. Because the microenvironment is critical for normal hematopoietic stem/progenitor cell function, we aimed to determine whether alterations in the TIMP/MMP balance impact upon normal hematopoiesis in mice. We have used both overexpression and knockout mouse models to determine whether early hematopoiesis is susceptible to potentially pathologic changes in TIMP/MMP level. These studies used TIMP-1^{-/-} mice and retroviral vectors co-expressing human TIMP-1 or TIMP-2 linked with the green fluorescent protein (GFP) transduced into bone marrow (BM) cells and transplanted into lethally irradiated recipient mice. Loss of TIMP-1 in knockout mice or retroviral overexpression of TIMP-1 or TIMP-2 did not alter hematopoietic stem/progenitor function during steady-state hematopoiesis. Surprisingly, even when applying hematopoietic stress through mobilization, chemotaxis, or myelosuppression, murine hematopoiesis was not adversely affected by TIMP-1 or TIMP-2 level. We conclude that TIMP/MMP balance alone does not exert significant influence on blood cell development and homeostasis. An important corollary of these studies is that specific modulation using MMP inhibitors for cancer or immunologic therapy is unlikely to have adverse hematopoietic side effects.

INTRODUCTION

The tissue inhibitors of matrix metalloproteinase (TIMP)s are natural inhibitors of matrix metalloproteinases (MMP)s and act by tightly binding the MMP in a 1:1 stoichiometric ratio. This interaction occurs through an MMP binding domain within the N-terminal region of the protein (1–126 amino acids referred to as N-TIMP)[1;2] and this binding is dependent on several cysteine residues and a disulfide bridge that stabilizes the protein[3]. MMP substrates

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include many components of the microenvironment of hematopoietic stem cells (HSC) and progenitors, such as collagens, laminins, and fibronectin[4]. Therefore, TIMPs and MMPs in hematopoiesis may be able to modulate extracellular matrix interactions of HSCs and early progenitors.

The bone marrow (BM) microenvironment is thought to be a critical regulator of HSC survival, resulting from direct interaction with HSCs and through soluble factors secreted from the stromal cells[5]. Homing of HSCs to the BM niche is dependent on $\beta 1$ integrins such as the very late antigen (VLA) integrin $\alpha 4\beta 1$ (VLA-4; binds to CS-I or VCAM-1)[6–8] and $\alpha 5\beta 1$ (VLA-5; binds to classical fibronectin RGD)[9;10]. Under steady-state conditions, TIMP and MMP expression in the BM is low[11;12], however growth factor stimulation results in a proteolytic microenvironment favoring mobilization[13]. This is due in part to increased secretion of neutrophil gelatinase B (MMP-9)[14;15]. Gelatinase activity of MMP-2 and MMP-9 is increased in the BM during hematopoietic stress and mice lacking MMP-9 are susceptible to myelosuppression induced by chemotherapeutic agents[16].

Tissue inhibitor of matrix metalloproteinase (TIMP)-1 and TIMP-2 have been identified in various myeloid cell types including platelets, megakaryocytes, and BM fibroblasts[17] and are associated with adaptive immunity, inflammatory responses, and chronic myeloproliferative disorders (MPD). The effects of growth factors on TIMP and MMP expression are cell type dependent. For example, growth factors such as stem cell factor (SCF) decrease MMP-9 in mast cells[18], while IL-6 can increase secretion of MMP-9 and TIMP-1 in non-Hodgkin's lymphoma[19]. In human lymphoid T and B cell lines, IL-6 stimulation increased MMP-9 secretion without having an effect on TIMP-1[20]. In monocyte/macrophage differentiation, growth factors such as IL-1 β and TNF α are upregulated as part of the inflammatory response. MMP-9 is also upregulated to promote the extravasation and migration of the macrophage to the site of infection and to aid in clearing debris. Coordinate upregulation of TIMP-1 at early stages of the inflammatory response can be mediated by signaling receptors with strong preference for STAT3 activation[21], such as IL-6. At later stages as part of the anti-inflammatory response, IL-10 generated from B cells and macrophages stimulates maximal TIMP-1 expression[22;23] also via STAT3 activation[24].

In mice, human TIMP-1 overexpression has been reported to cause phenotypes in non-hematopoietic tissues. For example, roles in development and cancer progression have been validated in human TIMP-1 transgenic mice. Overexpression of human TIMP-1 from the human β -actin promoter in transgenic mice showed reduced length of E6.5 decidua[25]. We have also shown that human TIMP-1 expression in Burkitt's lymphoma mouse xenografts caused increased NK1.1 and decreased Gr-1 levels[26]. Roles for TIMP-1 in pathologic models have been reported using transgenic approaches for the study of TIMP-1 effects in the areas of tumor growth and metastasis[27–32] and inflammation. In these models TIMP-1 transgene expression was driven under different tissue specific promoters resulting in differing levels of circulating plasma human TIMP-1 that cross-reacts with murine MMPs. In mice where TIMP-1 was under control of ubiquitous β -actin promoter/enhancer, circulating TIMP-1 was about 40 ng/mL[33] while a liver specific albumin promoter/enhancer resulted in a higher plasma level of TIMP-1 (~500 ng/mL)[34;35]. For comparison, the normal plasma level of murine TIMP-1 is 1.25–3.4 ng/mL (Quantikine mouse TIMP-1 ELISA kit, R&D Systems). Mouse *Timp-1* driven by the murine MHC class I H2 promoter showed reduced formation of hepatocellular carcinomas[36] and reduced metastasis of T-cell lymphoma[37].

While less is known about transgenic TIMP-2 expression, TIMP-2 plays a unique role in preventing endothelial cell proliferation and is being explored for anti-angiogenic therapy [38]. Many examples in the literature can be found describing growth factor-like activities for TIMPs in a wide range of cell types from fibroblasts to lymphocytes and erythrocytes. Recent

studies have identified the tetraspanin family molecule CD63 as the TIMP-1 interacting protein responsible for effects on integrin mediated signaling in MCF10A cells[39]. Our previous study showed growth altering function on mouse M1 myeloblasts[40]. However no studies have addressed the possible role of TIMP-1 or TIMP-2 as a growth factor in hematopoiesis. Therefore, in this study we used opposing approaches to determine whether TIMP-1 or TIMP-2 levels have influence on hematopoiesis.

METHODS

Animals

The C57BL/6J (BL/6, CD45.2) mice and the congenic strains B6.C-Tyrc H1^b Hbb^d/ByJ (HW80, CD45.2) and B6.SJL-Ptprc^aPep3^b/BoyJ (CD45.1) were obtained from the Jackson Laboratory (Bar Harbor, ME). The occurrence of polymorphic changes at the CD45 (Ly5) locus facilitates tracking of transplanted donor cells. Homozygous TIMP-1 deficient C57BL/6 mice were originally developed by Dr. Paul Soloway (Roswell Park Cancer Institute, Buffalo, NY)[41] and bred in our facility. The presence of TIMP-1 WT or KO allele was monitored by PCR genotyping using either TIMP-1 or Neo primer pairs. A product of the wild-type allele is ~400 bp and a product of the KO allele is ~480 bp.

Timp-1Ex2: 5'-GTCATAAGGGCTAAATTCATGGG-3';

Timp-1Ex3: 5'-ACTCTTCACTGCGGTTCTGGGAC-3';

Neo1: 5'-ATGATTGAACAAGATGGATTGCAC-3';

Neo2: 5'-TTCGTCCAGATCATCTGATCGAC-3'

All mice were housed in a specific pathogen-free environment. All studies were approved by the Case Institutional Animal Care and Use Committee.

Bone marrow transduction and transplantation

Donor mice (30–50 mice C57BL/6 ~ 10–15 weeks of age) were treated with intraperitoneal injection of 5-fluorouracil (American Pharmaceutical Partners, Inc., Schaumburg, IL) at 150 mg/kg 3 days before harvesting BM cells. BM was harvested from both hind limbs (tibiae and femurs) and BM cells cultured in stimulation media (IMDM with 15% FBS (HyClone, Logan, UT) and 2% penicillin/streptomycin/amphotericin B supplemented with 50 ng/mL rmSCF, 20 ng/mL rmIL-3, and 50 ng/mL rhIL-6 (R&D Systems, Minneapolis, MN)) at 2×10^6 cells/mL. After 2 days of pre-stimulation the BM cells were plated over 15 Gy irradiated GP+E86 producer cells. An MSCV-based bicistronic retroviral vector containing human TIMP-1 cDNA in addition to GFP was transduced into BM cells in stimulation media supplemented with 6 μ g/mL polybrene (Sigma-Aldrich Corp., St. Louis, MO) at 1×10^6 cells/mL. After 2 days of co-culture the BM cells were harvested and resuspended in PBS with 2% FBS at $2\text{--}5 \times 10^6$ cells/mL. Heparin (Sigma Chemical, St. Louis, MO) was added at 0.1 mg/mL and the cells were injected via the lateral tail vein into lethally irradiated (9.5–11 Gy) recipient mice (CD45.1). An aliquot of transduced cells was used for assessing the transduction efficiency by flow cytometry and to perform the colony assay. Secondary transplants were performed by harvesting BM cells from mice that had been transplanted 16 weeks earlier. BM cells were injected into lethally-irradiated secondary hosts at a donor to recipient ratio of 1:5.

Colony-forming unit (CFU-C) assay

BM cells (1×10^5 freshly harvested or 1×10^4 cultured cells) were plated in 3 mL methylcellulose media (MethoCult M3334; Stem Cell Technologies, Vancouver, BC, Canada) supplemented with 50 ng/mL rmSCF, 20 ng/mL rmIL-3 and 50 ng/mL rhIL-6. Duplicates of 3 independent cultures were plated for each sample and they placed in a humidified chamber with 5% CO₂

at 37 °C. After 7 days the colonies containing at least 30 cells were scored. The blood samples were lysed for 10 minutes at room temperature in hypotonic solution (0.155 M NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA) to remove red blood cells. The white blood cells were resuspended in PBS/2% FBS of original blood volume. For CFU-C assay 50 and 100 µl aliquots were plated.

Flow cytometry analysis

BM or peripheral blood cells were analyzed for specific antigens or GFP expression by flow cytometry. To assess the percentage of CD45.2 donor engraftment in CD45.1 recipient mice, the cells were stained with the biotinylated anti-CD45.2 antibody (CD45.2, 104) followed by secondary staining with phycoerythrin (PE)-conjugated streptavidin. When a multilineage analysis was required, the staining with a cocktail of PE-conjugated lineage specific antibodies was performed. The lineage markers included Gr-1, B220, CD4 (L3T4), TER-119. For competitive engraftment analyses peripheral blood leukocytes of recipient mice were stained with FITC-conjugated CD45.2 (CD45.2, 104) and PE-conjugated CD45.1 (CD45.1, A20) antibodies. All antibodies for these studies were obtained from BD Pharmingen (San Diego, CA). Cells were then analyzed on an LSR I flow cytometer (BD Biosciences, San Jose, CA) using CellQuest software.

ELISA

Protein concentration of human TIMP-1 either in plasma or BM extracellular extract was determined using ELISA kit. Plasma was obtained from blood after centrifugation for 10 minutes at 2000 × g. BM extracellular extract was obtained from BM flushed with ~800 µL PBS/hind limb (1 femur + 1 tibia) after centrifugation for 10 minutes at 2000 × g. The samples were stored at -80 °C until analysis by ELISA. Plasma was analyzed at 100–1000-fold dilution and BM extracellular extract at 10–50-fold dilution. ELISA was performed according to the manufacturer's instructions using a kit that recognized both the free and MMP-bound forms of TIMP-1 (Calbiochem, San Diego, CA). For assay of human TIMP-2 and human MMP-9 similar kits were used (R&D Systems, Minneapolis, MN; Calbiochem, San Diego, CA, respectively). The concentration of mouse TIMP-1 was measured with mouse specific TIMP-1 kit (R&D Systems, Minneapolis, MN).

Zymogram and reverse zymogram

Gelatinase activity was assayed by zymogram in a gelatin containing gel as previously described[42]. Gelatinase activity appeared as a clear (unstained) band of degraded gelatin on blue background of Coomassie stained gel. Metalloproteinase inhibitory activity was assayed by reverse zymogram as previously described[43]. TIMPs were visualized as bands of nondegraded gelatin staining positive with Coomassie Blue.

5-FU and G-CSF treatment of transplanted mice

Mice that were previously transplanted and fully reconstituted with retrovirally-transduced donor hematopoietic cells were treated with either i.p. injection of 5-FU (150 mg/kg; American Pharmaceutical Partners, Inc., Schaumburg, IL) or s.q. injections for 5 days with recombinant human granulocyte-colony stimulating factor (G-CSF, 200 µg/kg/d; Neupogen, Amgen, Inc., Thousand Oaks, CA). All recipient mice in each group were transplanted with the same pool of donor BM cells and mobilization was performed at time from 2 to 6 months later.

Transmigration assay

Transmigration assay was performed in Costar transwell plates (24-well, diameter 6.5 mm, pore size 5 µm, Corning International, Corning, NY) through the membrane coated with human fibronectin. The lower chamber contained 600 µl RPMI/1% BSA media with or without 100 ng/ml SDF-1α (R&D Systems, Minneapolis, MN). The upper chamber (insert) was loaded with

1×10^6 BM cells in 100 μ l RPMI/1% BSA. After 5 h incubation at 37 °C the migrated cells were counted. To calculate the progenitor migration rate both the original BM cells and the migrated cells were plated in methylcellulose medium to assess CFU-C frequency.

RESULTS

Loss of TIMP-1 does not impair hematopoietic stem/progenitor cell function

To determine whether lack of TIMP-1 would have any effects on HSC or progenitor cell function, TIMP-1^{-/-} mice on the C57BL/6 background were examined. BM colony-forming ability *in vitro* in methylcellulose media under limiting concentrations of cytokine cocktail (SCF-IL3-IL6) was first checked (Fig. 1A). The Epo concentration was kept the same in all CFU-C assays. We did not see any significant difference in frequency of total progenitors between wild-type and TIMP-1^{-/-} BM cells at standard or reduced concentrations of SCF, IL-3 and IL-6 (50, 20, 50 ng/mL, respectively). This indicates that TIMP-1 does not play a cell intrinsic role in growth response of myeloid CFU-C.

To investigate potential differences in HSC biology where the microenvironment could be altered, we compared engraftment capability in a direct competitive transplant setting (Fig. 1B). CD45.2 BM cells were harvested from TIMP-1^{-/-} and wild-type mice and were mixed in the same mouse equivalent ratio with CD45.1 BM cells and transplanted into lethally irradiated CD45.1 recipients (10 mice total per genotype). After 8–12 weeks of engraftment, CD45 chimerism in peripheral blood leukocytes (PBL) was measured by flow cytometry to assess the contribution from each donor to overall hematopoiesis. There were no differences in competitive repopulation noted between TIMP-1^{-/-} HSC and wild-type in primary and secondary recipients in two separate transplant experiments. Furthermore, loss of TIMP-1 in TIMP-1^{-/-} mice did not impact upon G-CSF induced mobilization (Fig. 1C).

Elevated TIMP or MMP expression does not adversely alter hematopoietic stem cell repopulating activity

For overexpression studies we designed retroviral vectors (Fig. 2A). To transduce primary murine BM cells, we used GP+E86 based retroviral producer cells. The retroviral vector backbone was based on the murine stem cell virus (MSCV) driving expression of a human TIMP-1 cDNA. An internal ribosomal entry site (IRES) was also included to permit expression of the enhanced green fluorescent protein (GFP) marker. Ecotropic producer cells were generated using these constructs and previously characterized for TIMP-1 expression[44]. As expected, using this standard approach we were able to achieve high levels of gene transfer into BM cells and CFU-C progenitors (81±17% for the IR-GFP and 86±7% for the TIMP-1 expressing vector). TIMP-1 protein expression and secretion from the target cells was confirmed by human TIMP-1 specific ELISA using BM cell conditioned media obtained prior to transplant. The transduced BM cells expressed and secreted TIMP-1 at the level of 24.5 ±11.7 ng/1×10⁶ cells (Fig. 2B). As a control, no detectable human TIMP-1 could be found in BM cells transduced with the MSCV-IRES-GFP control vector. TIMP-1 expression did not affect the frequency of any subtype of myeloid progenitor colony at normal or reduced concentration of cytokines (Fig. 2C).

The transduced BM cells were transplanted into lethally irradiated (9.5–11 Gy) recipient mice (CD45.1) in 8 separate transplant experiments (45 total IRES-GFP mice and 66 total TIMP-1 mice). At this point the engraftment of donor cells was assessed by their contribution to overall hematopoiesis. The level of GFP marking in PBL was 71±20% for the IRES-GFP control vector and 62±26% for the TIMP-1 expressing vector as measured by flow cytometry (Fig. 3A). The engrafted transduced cells were able to produce human TIMP-1 at significant yet very variable levels in the plasma. The range of plasma concentrations was from 60–525 ng/mL (Fig. 3A)

and did not show any correlation with the gene transfer efficiency as indicated by the percentage of GFP⁺ PBL. Therefore GFP simply serves as a marker for transduced cells. After full reconstitution of hematopoiesis (8–12 weeks) the transplanted mice showed normal hematology (data not shown). The percentage of GFP⁺ PBL remained steady in the mice throughout the observation period. Histological analysis of these mice did not show any abnormalities in hematopoietic tissues. To assess whether HSC were transduced with the retroviral vectors, secondary transplants were performed. Secondary recipient mice were analyzed for donor engraftment, GFP marking, and TIMP-1 expression. Although GFP marking was still very high, circulating TIMP-1 levels were similar to the primary transplanted mice (data not shown). Analysis of BM extracellular fluid showed BM levels of human TIMP-1 (Fig. 3B) that were much higher than the endogenous levels of mouse TIMP-1 (Fig. 3C). As expected, the circulating levels of TIMP-1 were always higher than the BM levels when normalized for the cellularity.

To confirm enzyme function and extend analysis to TIMP-2, reverse zymogram analysis was performed. Results from these analyses are shown in Fig. 4A and provide evidence that the expressed TIMPs and MMPs are functional in retroviral producer cells. All cells expressed basal levels of TIMP-2, as well as low levels of TIMP-1. However, TIMP-2 ecotropic producer cells showed enhanced TIMP-2 expression over the basal level and a slight decrease in TIMP-1. The TIMP-1 ecotropic producer cells showed a marked increase in TIMP-1 expression with no change in TIMP-2. Zymogram analysis on MMP-9 producer cells also demonstrated gelatinase activity (Fig. 4B) as a zone of clearing in the gelatin-containing gel. These vectors were used to generate transplanted mice with high levels of expression in the peripheral blood as measured by ELISA (Fig. 4C). Like mice overexpressing TIMP-1, all mice overexpressing TIMP-2 and MMP-9 at ranges similar to the TIMP-1 vector showed normal peripheral blood counts (data not shown).

Overexpression of TIMP-1 or TIMP-2 does not alter bone marrow cell migration *in vitro* or mobilization *in vivo*

To test the role of TIMPs during proliferative stress hematopoiesis, we performed migration and hematopoietic progenitor mobilization experiments. Transmigration was performed using retrovirally-transduced BM cells expressing either IRES-GFP control or TIMP-1 or TIMP-2 (Fig. 5A). There was no effect of either TIMP-1 or TIMP-2 on the migration ability in response to an SDF-1 α chemotactic gradient.

The recruitment and mobilization of progenitors has been shown to involve matrix metalloproteinase (MMP)-9 and the release of membrane bound SCF from the extracellular matrix[45]. To test whether overexpression of TIMP-1, TIMP-2, or MMP-9 impacts upon mobilization of CFU-C, mice were treated with a mobilizing regimen. A dose of 200 μ g/kg/d of G-CSF was subcutaneously administered to cohorts of mice for 5 days. The frequency of myeloid progenitors in peripheral blood was assessed by CFU-C assay. Unmobilized mice had a low level of circulating progenitors (20–40 CFU-C/ml). Mice treated with G-CSF had CFU-C mobilized into the circulation at similar levels whether regardless of human TIMP-1 plasma level (range 66 – 131 ng/ml). Also, no significant differences were observed between groups overexpressing human MMP-9 (range 103–289 ng/ml) or TIMP-2 (range 20–370 ng/ml) compared to control mice (Fig. 5B). These results demonstrate an absence of effects on CFU-C mobilization by G-CSF despite high level TIMP expression in the mouse BM.

Overexpression of TIMP-1 or TIMP-2 does not alter recovery from 5-FU induced myelosuppression

To determine whether TIMP-1 or TIMP-2 overexpression causes defects in mice following a myeloablative hematopoietic stress, we treated mice with a single i.p. injection of 5-fluorouracil

at 150 mg/kg of body weight. The myelosuppression induced by 5-FU has been well characterized and leads to release of MMP-9 into the BM extracellular matrix. Fig. 6A shows the increase in gelatinase activity from BM of mice treated with 5-FU that peaks 2 to 3 days following treatment. Data collected from two independent cohorts of transplanted mice showed significant myeloablation as measured by hematocrit and absolute neutrophil count. In all mice regardless of TIMP-1, TIMP-2, or MMP-9 overexpression, hematopoietic recovery was complete by 3 weeks in both hematocrit (Hct) and total nucleated white blood cell (WBC) count (Fig. 6B).

DISCUSSION

In these studies we have extensively manipulated TIMP/MMP balance to determine the extent to which normal hematopoiesis is altered. We found that the absence of TIMP-1 does not impair or enhance normal hematopoiesis. TIMPs play the role of regulating MMPs to control maintain homeostasis. Therefore, other mechanisms that down regulate MMP-dependent responses may remain intact and not allow for an overly active hematopoietic response following transplantation induced myelosuppression. It is also likely that without hematopoietic stress to induce MMP release and activation, TIMP-1 is essentially without significant normal function in hematopoietic development. Our results are consistent with this finding and suggest that aberrant expression of TIMP or MMP may not lead to pathology unless combined with additional underlying conditions such as cancer or inflammation and its treatment.

To determine whether excess TIMP-1 can cause adverse BM function, we treated TIMP-1 overexpressing mice with G-CSF to induced hematopoietic progenitor mobilization. Release of MMP-9 along with other proteases from neutrophil granules has been proposed to mediate important functions during hematopoiesis. MMP-9 can release sequestered c-Kit ligand (stem cell factor; SCF) that is then available to bind to c-Kit and promote hematopoiesis or cleave SDF-1 resulting in mobilization. Mice lacking MMP-9 expression are hypersensitive to 5-FU [46] and die from prolonged myelosuppression. The observation that TIMP-1 overexpression did not attenuate G-CSF induced mobilization of CFU-C agrees with a report using MMP-9^{-/-} mice on the C57BL/6 background[47]. We also extended this analysis further by testing TIMP-2 overexpression. TIMP-2 was also not able to alter CFU-C mobilization capacity. TIMP-1 and TIMP-2 have similar but not identical substrate specificity for pro-MMP forms, but each should effectively bind to MMP-2 and MMP-9 which are believed to be the major isoforms involved in mobilization. Furthermore, our demonstration that migration is equally unaffected provides *in vitro* evidence supporting this conclusion. Our preliminary analyses of bone marrow from TIMP-2^{-/-} mice also indicated no effect on CFU-C number relative to wild-type littermate bone marrow (data not shown) and similar to TIMP-1^{-/-} bone marrow (Fig. 1A).

Since TIMP/MMP balance is difficult to measure *in situ* in the BM cavity, the exact amount actually present during steady-state in the BM is not known. As new molecular imaging technologies for imaging live mice are developed, this might become possible in the future. Our data with BM extracellular fluid as a surrogate allows comparison with plasma levels of circulating TIMP-1. It is known that TIMPs and MMPs are released from monocytes, neutrophils, and platelets. These mature cell types are not well represented in the BM under steady-state conditions. Their lower numbers and lack of activation may contribute in part to lower levels of secreted TIMP-1 in the BM extracellular fluid. Secretion of MMP-9 (gelatinase B) has been described in neutrophils to be dependent on an active secretion mechanism[48]. Our data indicate however that significant overexpression relative to mouse TIMP-1 can be achieved in the BM microenvironment.

TIMP-1 may inhibit regeneration of the hematopoietic vascular niche and/or other changes in the hematopoietic reconstitution that limit neutrophil emigration. In response to cytotoxic drugs that initiate translocation of intestinal microflora[49], it is possible that TIMP-1 may play roles that have not been uncovered through our studies. Clearly further studies will be required to investigate these possibilities. In our studies, the response to 5-FU was not altered and the recovery from the nadir was as rapid as wild-type mice. Notably, TIMP-1^{-/-} mice are hyper-resistant to bacterial infections due to loss of an inhibitory effect on neutrophils [50], suggesting that TIMP-1 normally put the brakes on host defense as part of the anti-inflammatory response. Recent demonstration of reduced neutrophilic infiltrates in *Francisella tularensis* infection result from MMP-9 deficiency, further support a role for MMP-9 in innate immune response [51].

In this study using the same retroviral vector that we previously described, we found no evidence for growth promoting activity of TIMP-1 or TIMP-2 at any stage of hematopoietic development. This was despite very high levels accumulating in blood plasma to levels much higher than typical cytokines. Since TIMPs may only be required in the presence of MMP activation, the extra TIMP activity does not confer MMP-independent effects on hematopoiesis. It should be noted that there is literature describing erythroid potentiating activity (EPA) for both TIMP-1 and TIMP-2. Like prior reports, we did observed modestly elevated BFU-E in some CFU-C cultures (data not shown). However, *in vivo* the hematocrit of mice expression TIMP-1 or TIMP-2 was not increased above control levels, so the physiological significance of EPA remains unclear.

Overall the results of these experiments are somewhat surprising. How could the TIMP/MMP balance not be critical for hematopoietic function? Since neither TIMP-1 or TIMP-2 were capable of dominantly interfering with hematopoietic stem/progenitor recruitment and mobilization, it is possible that redundancy exists. This redundancy may be due to additional MMP activity for which TIMP-1 or TIMP-2 cannot act. However, since MMP-2 and MMP-9 are the strongest candidates for hematopoietic regulation, this is not likely. Perhaps development and testing of compound TIMP-1/TIMP-2 double mutant mice on the C57BL/6 background would provide new information. Alternatively, it is possible that other stromally-derived protease/protease inhibitors can fulfill overlapping roles in hematopoietic regulation and that even combined TIMP-1/TIMP-2 deletion would not yield significant defects in hematopoiesis. It is known that the plasminogen system and additional proteases such as neutrophil elastase and cathepsin D are also potentially capable of redundant functions.

Since the mice are viable and do not have overt hematopoietic defects, the animal models described here may provide useful tools for further study in the context of inflammatory or myeloproliferative disease. Skewing of the maturation stage of the myeloid lineage toward the terminally differentiated neutrophil or macrophage may further promote release of TIMP or MMP in the BM. In addition, these studies provide the strongest evidence that experimental drugs targeting TIMP or MMP could be safe in terms of hematologic toxicity. Even under conditions of hematopoietic stress, the TIMP/MMP balance could be significantly altered with side effects, potentially improving the therapeutic benefit for treatment of blood diseases.

Abbreviations

TIMP, tissue inhibitor of matrix metalloproteinase; MMP, matrix metalloproteinase; BM, bone marrow; HSC, hematopoietic stem cell; MSCV, murine stem cell virus; PBL, peripheral blood leukocyte.

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Figure 1. TIMP-1 deficiency does not alter myeloid progenitor frequency in limiting cytokine concentrations or HSC competitive repopulating function

A. Freshly isolated BM cells (1×10^5) from TIMP-1 WT or TIMP-1 KO mice, respectively were plated in methylcellulose media containing recombinant human Epo (3 U/mL) supplemented with recombinant murine SCF (50 ng/mL) – recombinant murine IL-3 (20 ng/mL) – recombinant human IL-6 (50 ng/mL) (= 1x) or 10x- and 50x-lower concentration, respectively. After 7 days colonies comprising >30 cells were scored. Bars represent average of 3 independent cultures. **B.** A mouse equivalent of pooled BM cells from TIMP-1 KO mice ($\sim 5 \times 10^6$ cells) was mixed with a mouse equivalent of pooled BM cells from CD45.1 donor mice and co-injected into 5 recipient mice (CD45.1). As a control a mouse equivalent of pooled BM

cells from TIMP-1 WT mice was mixed with a mouse equivalent of pooled BM cells from CD45.1 mice and injected into 5 recipient mice (CD45.1). The bars represent the proportion of either TIMP-1 KO or TIMP-1 WT (CD45.2) derived PBL 2 months after injection combined from two independent experiments. **C.** TIMP-1 KO and TIMP-1 WT mice were treated with s.q. injection of G-CSF (200 μ g/kg/day) for 5 days. The frequency of circulating progenitors was assessed by CFU-C assay. Untreated mice as controls are also included. The frequency is expressed per ml of blood. As a control C57BL/6 mice (WT) were mobilized at 460–940 CFU-C/ml. Error bars represent the standard deviation.

Figure 2. High levels of retroviral TIMP-1 gene transfer in BM cells does not alter BM CFU frequency

A. A map of the retroviral vector expressing human TIMP-1 cDNA used for transduction of BM cells. A parental vector without any insert was included as a control. **B.** The transduced BM cells were analyzed for GFP expression by flow cytometry. In addition conditioned media was prepared from the transduced cells to assess the level of expression and secretion of TIMP-1 by ELISA. Bars represent the percentage of GFP positive cells (transduction efficiency) and specific TIMP-1 expression by the transduced cells. **C.** Cultured BM cells (1×10^4) were transduced with either the MSCV-TIMP-1-IRES-GFP or MSCV-IRES-GFP retroviral vector and plated in methylcellulose media containing recombinant human Epo (3 U/mL) supplemented with recombinant murine SCF (50 ng/mL) – recombinant murine IL-3 (20 ng/mL) – recombinant human IL-6 (50 ng/mL) (= 1×) or 10×- and 50×-lower concentration, respectively. After 7 days, colonies comprising >30 cells were scored. Bars represent the average of progenitor frequency in 3 independent cultures.

Figure 3. TIMP-1 overexpression in bone marrow transplant primary recipients

A. Flow cytometry analysis of GFP expression in PBL and ELISA analysis of mouse plasma obtained from primary recipient TIMP-1 transplanted mice. Mice were analyzed 2 month after BM transplant with transduced BM cells (with MSCV-TIMP1-IRES-GFP or MSCV-IRES-GFP retroviral vector, respectively). The bars represent individual mice; the last bar in the group indicating the average with the standard deviation. Concentration of TIMP-1 protein in plasma and BM extracellular extract was measured by human TIMP-1 specific ELISA. Data is shown for individual mice as bars on the X-axis. **B.** Concentration of TIMP-1 protein in plasma and BM extracellular extract was measured by a mouse TIMP-1 specific ELISA and is shown for individual mice. Note that the mice in panel B and C are the same and this allows for comparison of the relative levels of human vs. mouse TIMP-1 in the same BM sample. The concentration in blood was determined using the hematocrit as a conversion factor and then

the concentration was normalized per 1×10^6 cells as calculated from the WBC count. Normalized concentration in BM extracellular extract was calculated from cellularity obtained from flushed BM.

Figure 4. Retroviral TIMP-1, TIMP-2, or MMP-9 have functional gelatinase inhibitory/gelatinase activity and are highly expressed in transplanted mice

A. Reverse zymogram analysis of conditioned media from ecotropic producers used for transduction of BM cells. Recombinant TIMP-2 was used as a positive control. Signals corresponding to specific TIMP activity are marked by arrows on the side. **B.** Zymogram analysis for detection of gelatinase activity of conditioned media from MMP-9 producers. Gelatinase B specific activity corresponding to MMP-9 protein is marked by an arrow. **C.** Expression of transgene in transplanted mice. The secreted level of protein was assayed by ELISA in plasma from peripheral blood 2 months after transplantation.

Figure 5. Overexpression of TIMP-1 or TIMP-2 does not alter bone marrow migration in vitro or mobilization in vivo

A. The BM cells were harvested from transplanted mice and were subjected to transmigration assay in a transwell setting towards a 100 ng/ml SDF-1 α gradient for 5 h. Based on progenitor frequency in total BM cells and the migrated BM cells the transmigration rate was calculated. Data is presented as % of migrated GFP⁺ CFU-C and the error bars represent standard deviation. **B.** Cohorts of GFP, TIMP-1, TIMP-2 and MMP-9 mice were mobilized with 200 μ g/kg/d G-CSF (s.q. injection) for 5 days and the frequency of circulating progenitors was assessed by CFU-C assay. The frequency is expressed per ml of blood and error bars represent standard deviation. The IRES-GFP control (GFP) BM transplant mice were mobilized at 460–12,000 CFU-C/ml. Due to the variation in absolute mobilization level from experiment to experiment, CFU-C/ml was normalized to the GFP control mice (defined as 100%) for each individual experiment.

Figure 6. Overexpression of TIMP-1, TIMP-2 or MMP-9 does not alter recovery from 5-FU induced myelosuppression

A. The mice were treated with a single i.p. injection of 5-FU (150 mg/kg) and in some mice gelatinase activity in the BM was quantitated by zymogram using gelatin as substrate. **B.** Following 5-FU treatment hematology analysis was performed 7, 14, and 21 days later. Hematocrit (Hct) and total white blood cell (WBC) count are presented for each cohort separately.