Prominent role of TGF-β1 in thrombopoietin-induced myelofibrosis in mice

Short title: Role of TGF-β1 in myelofibrosis

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Abstract

Several studies suggest an implication of TGF-β1 in the promotion of myelofibrosis associated with hematopoietic malignancies, but the involvement of this cytokine is not fully investigated. To test directly the impact of TGF-β1 in the pathogenesis of myelofibrosis, bone marrow stem cells from homozygous TGF-β1 null (TGF-β1−/−) and wild-type (WT) littermates were infected with a retrovirus encoding the murine TPO protein and engrafted into lethally irradiated wild-type hosts for long-term reconstitution. Over the 4 months of follow-up, TPO levels in plasma were markedly elevated in both groups of mice and animals typically developed a myeloproliferative syndrome characterized by thrombocytosis, leukocytosis, splenomegaly, increased numbers of progenitors in blood and extramedullary hematopoiesis. Severe fibrosis was observed in spleen and marrow from all the mice engrafted with WT cells. In contrast, none of the mice repopulated with TGF-β1−/− cells (chimerism > 70%) showed deposition of reticulin fibers at any time during the follow-up. In accordance with the development of fibrosis, latent TGF-β1 levels in plasma and extracellular fluid of the spleen from mice engrafted with WT cells were increased 6-fold and 4-fold, respectively, over levels found in normal hosts, whereas no increase over baseline levels could be demonstrated in animals transplanted with TGF-β1−/− cells. These data provide evidence that TGF-β1 produced by hematopoietic cells is pivotal for the pathogenesis of myelofibrosis that develops in mice with TPO overexpression.

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Introduction

Myelofibrosis is a prominent clinical feature of several hematopoietic disorders, and particularly in idiopathic myelofibrosis. It occurs as a cytokine-mediated secondary response to a clonal malignant event originating in a pluripotent stem cell and is characterized by excessive deposits of extracellular matrix proteins, neo-angiogenesis, and, in severe cases, osteosclerosis. In vivo and in vitro studies have involved several cytokines, such as TGF-β1, PDGF or bFGF, in the development of myelofibrosis. Among these growth factors, the pleiotropic cytokine TGF-β1 has received attention since TGF-β1 potently stimulates fibroblasts to produce extracellular matrix and cell adhesion proteins and enhances the expression of proteases that inhibit enzymes involved in the degradation of the extracellular matrix. TGF-β1 is secreted by numerous cell types in the body, but several lines of evidences have suggested a crucial role of megakaryocytes in myelofibrosis induction. First, TGF-β1 is the most abundant isoform found in megakaryocytes and platelets. Second, on marrow biopsies from patients with idiopathic myelofibrosis, megakaryocytes are often found in close proximity or engulf into reticulin fibers and, third, acute myelofibrosis is often associated with megakaryoblastic leukemia. Despite these constitant evidences, it remains unclear whether TGF-β1 alone promotes myelofibrosis or whether it acts in concert with others fibrogenic cytokines to induce the aberrant stromal reaction. Such a demonstration has been hampered by the lack of an experimental model allowing to address this issue.

Over the past years, animal models that recapitulates the clinical features of human idiopathic myelofibrosis have been reported. In vivo administration of suprapharmacological doses of TPO, the physiological regulator of platelet production, resulted in megakaryocyte hyperplasia associated with a densification of the reticulin network. Mice permanently exposed to high doses of TPO delivered through retroviral infection of hematopoietic stem cells developed a myeloproliferative syndrome with a prominent proliferation of megakaryocytes and leukocytes, extramedullary hematopoiesis, and invariably splenic and medullary fibrosis and osteosclerosis. In these experimental models, it has been hypothesized that TGF-β1 had an important role in the development of myelofibrosis since elevated levels were detected in plasma, platelet extracts and marrow fluids at time of myelofibrosis development which gradually decreased with fibrosis normalization. However, increased levels of PDGF were also demonstrated in plasma from TPO-
overexpressing mice suggesting that both TGF-β1 and PDGF, or possibly other cytokines, could influence the fibrogenic and osteogenic responses.

To clarify further the pathological effect of TGF-β1 in mice with megakaryocyte hyperplasia, we long-term repopulated lethally irradiated wild-type hosts with either TGF-β1−/− or wild-type (WT) hematopoietic stem cells engineered to overexpress the murine TPO protein via a retroviral-mediated gene transfer. TPO elevation in plasma was similar in both groups of mice and all animals invariably developed a myeloproliferative syndrome. However, while myelofibrosis occurred in all the mice engrafted with WT cells, no deposition of reticulin was seen in mice reconstituted with a majority of TGF-β1+/− cells. Our data provide a direct demonstration that TGF-β1 released by hematopoietic cells plays a major impact in the pathophysiology of myelofibrosis.
Materials and methods

Mice

Heterozygote TGF-β1 breeders with a mixed Sv129 x CF-1 genetic background were provided by T. Doetschman (Cincinnati, OH). Mice were bred in our animal facility under specific pathogen-free conditions. Since 100% of homozygote TGF-β1−/− animals die before weaning, experiments were performed with cells obtained from 14-16 day-old male and female TGF-β1−/− or WT littermates. Lethally irradiated (10 Gy delivered by an X-ray apparatus) male and female wild-type mice (8 to 10 week-old) were used as hosts. For engraftment, irradiated host were injected via the retro-orbital sinus with 4 to 6 x 10^6 lineage-negative (Lin−) cells exposed to the MIGR-TPO virus.

PCR genotyping

DNA was extracted from distal tail segments from 12 day-old pups using standard techniques. For genotyping, 3 primers were used: primer A: 5’- GAGAAGAACTGCTGTCG 3’; primer B: 5’-GTGTCCAGGCTCCAAATATAGG-3’ and primer C: 5’-GCCGAGAAAGTATCCATCAT-3’. Primers A and B amplify a 142 bp product from the wild-type allele; primers B and C amplify a 500 bp product from the targeted allele. PCR conditions were as previously described.

Generation of the retrovirus

A full-length murine TPO cDNA was cloned upstream from the IRES of the MSCV-IRES-GFP (MIGR) retrovirus. Infectious defective virions were transiently produced by transfection of the 293 EBNA cell line with 3 plasmids: pCMV gag-pol, pCMV-VSV-G (vesicular stomatite virus envelope glycoprotein) both provided by J Morgenstein, Cambridge, MA) and the MIGR-TPO-GFP construct. Briefly, 293 EBNA cells were seeded at a concentration of 10^6 cells/well in 6 wells plates (Costar, Dutscher, France). The next day, 0.5 µg of each plasmid was cotransfected using Exgen reagent (Euromedex, Mundolsheim, France) according to the manufacturer’s recommendations. Supernatants were collected after 48, 72 and 96 h, and concentrated 20-fold over an amicon membrane (Centricon Plus-80, Millipore). Viral titers were determined by limiting dilution assay on NIH 3T3 cells. GFP fluorescence was analyzed by flow cytometry. Virus stocks containing 10^7 infectious particles/mL or more were used to infect the Lin− cell populations isolated from donors’ marrow.

Marrow cell preparation and infection
Single-cell suspensions prepared from femurs and tibiae were enriched for progenitors by immunomagnetic selection using a cocktail of CD45/B220 (clone RA3-6B2), CD4 (clone GK.1.5), CD5 (clone 53-7.3), Ly-6/GR1 (clone RB6-8C5), CD11/Mac-1 (clone M1/70) and TER 119 monoclonal antibodies (Pharmigen, San Diego, CA). Cells were incubated with immunomagnetic beads (Dynabeads M-450; Oslo, Norway) at a bead:cell ratio of approximately 4:1. To check for purity, the lineage-negative (Lin⁻) fraction was stained with a sheep anti-rat kappa light chain phycoerythrin-conjugated IgG and analyzed by flow cytometry. Routinely, purity ranged from 80 to 90%.

The Lin⁻ fraction obtained from WT mice was prestimulated during 24 h in alpha-Minimal Essential medium (Sigma Aldrich, Saint Quentin Fallavier, France) containing 10% heat-inactivated fetal bovine serum (FBS) and 5 recombinant cytokines (mu-FLT3-L, 20 ng/mL; mu-SCF, 25 ng/mL; mu-IL-3, 100 U/mL; mu-TPO, 10 ng/mL and mu-IL-6, 10 ng/mL). All cytokines were purchased from R&D (Oxon, UK). The Lin⁻ fraction obtained from TGF-β1⁻/⁻ mice was not subjected to prestimulation. Cells (1 x 10⁶) were incubated with 1 mL of the MIGR-TPO virus in the presence of the 5 cytokines for 24 h and 4 µg/mL polybrene (Sigma, St Louis, MO). Cells were centrifuged and incubated for another 24 h in fresh virus stock, fresh cytokines and polybrene. An aliquot was used for colony forming cell (CFC) assays; the remaining were inoculated into lethally irradiated hosts in a ratio of 3 donors per one recipient.

**In vitro clonogenic progenitor assay**

The frequency of CFC in the Lin⁻ fraction immediately after infection, in spleen, marrow and blood of engrafted animals was assessed in methylcellulose culture (Myelocult M3134; Stem Cell Technologies, Vancouver, Canada) containing 20% FBS and recombinant mu-IL-3 (100 U/mL), mu-TPO (10 ng/mL), mu-SCF (50 ng/mL) and hu-EPO (2 U/mL). Seeding densities were 5,000 cells/mL for the Lin⁻ fraction or 1 x 10⁵ cells/mL for spleen, marrow and blood mononuclear cells. All cultures were plated in triplicate and incubated at 37°C in a humidified incubator containing 5% CO2 in air. Colonies (> 50 cells) were scored at day 7 under an inverted microscope and randomly picked for PCR analysis.

**Analysis of engraftment and gene transfer**

To evaluate chimerism in hosts engrafted with TGF-β1⁻/⁻ cells, CFC-derived colonies from the marrow were analyzed by PCR using the TGF-β1 primers indicated above. To assess gene transfer efficiency, marrow-derived colonies from mice transplanted with TGF-β1⁻/⁻ or WT
cells were analyzed by PCR to detect the integrated retroviral sequence. Primers corresponding to the TPO cDNA were: sens 5’-ACTTTAGCCTGGAGAATGGAAA-3’ and antisens 5’-CCAGGA GTAATCTTGACTCTGA-3’ allowing the amplification of a 499 bp product. Actin was used as an internal control: sens 5’-GTACCACAGGCATTGTGATG-3’ and antisens 5’-GCAACATAGCACAGCTTCTC-3’. Thirty colonies were individually deposited into Eppendorf tubes containing 10 µL of lysis buffer (10mMol/L Tris hydrochloride (pH 8.3), 2 mMol/L MgCl2, 50 mMol/L KCl, 0.45% Tween 20) and 1 mg/mL proteinase K. Samples that failed to show a PCR product with actin were not included in the calculation of chimerism or gene transfer efficiency.

**Hematology, histopathology and immunohistochemistry**

Retro-orbital venous blood was sampled in citrated tubes. Numbers of nucleated cells and platelets, hematocrit values and differential cell counts were determined with a coulter calibrated for mouse blood (MS9, Schloessing Melet, Cergy-Pontoise, France). Platelet poor plasma (PPP) was prepared and stored at –20°C for determination of TPO and TGF-β1 levels. Tissues were fixed in Glyo-Fixx fixative (CML, Nemours, France) and embedded in paraffin. Sections (4-5 µm) were stained with hematoxylin/eosin, periodic acid Schiff and Giemsa for overall cytology. Reticulin fibers were revealed by silver staining according to Gordon-Sweet. Immunohistochemistry on spleen sections was performed with an anti-TGF-β monoclonal antibody (MAB 1835, clone 1D11, R&D Systems, Oxon, UK) used at 150 mg/mL. Antibody reactivity was revealed with streptavidin APAP (alkaline phosphatase anti-alkaline phosphatase) and Fast Red TR as a chromogene (DAKO, Trappes, France).

**TPO and TGF-β1 quantification**

TPO levels in platelet poor plasma (PPP) were determined with an ELISA (murine TPO Quantikine Kit, R&D Systems, Oxon, UK) according to manufacturers’ instructions. The sensitivity limit of the assay was 62.5 picogr/mL. The human TGF-β1 immunoassay (R&D Systems) which detects only active forms of TGF-β1 was used for determination of TGF-β1 levels in PPP, platelet extracts and extracellular fluid of spleens. Samples were prepared with a slight modification of the reported procedure. 20 Briefly, 500 µL of whole blood was collected on 500 µL citrated Hanks buffered saline solution (HBSS, Sigma Aldrich) and centrifuged at 200 g to prepared platelet rich plasma. Platelets were pelleted (2,000 g for 10 min), suspended in 200 µL HBSS and counted. An aliquot containing 4 x 10^8 platelets was suspended in a final volume of 500 µL HBSS and subjected to 3 cycles of freeze-thawing. Samples were centrifuged (12,000 g for 5 min) and platelet extracts were collected. An
aliquot of the spleen (100 mg) was gently disrupted in 1 mL HBSS, samples were centrifuged (1,000 g for 10 min) and supernatants were collected. All samples were assayed before (active TGF-β1) and after acidification (latent forms). For acidification, the protocol recommended by manufacturers was followed. The sensitivity of the assay was 62,5 picogr/mL.

**Statistical analysis**
The results are presented as mean ± standard deviation (SD). The data were analysed with the two tailed Student’s t-test.
Results

Engraftment with virus-infected TGF-β1+/− and TGF-β1+/+ marrow cells

To assess the contribution of TGF-β1 in myelofibrosis, Lin− cells from TGF-β1+/− pups and their WT littermates were infected with the MIGR-TPO retrovirus and engrafted into lethally irradiated wild-type hosts for long-term reconstitution. Whatever the donor genotype, recovery of the Lin− fraction was routinely around 5% with a mean purity of 80% (78%-90%; n = 12). To ensure efficient transduction in primitive hematopoietic stem cells, the Lin− fraction from WT donors was prestimulated in vitro during 24 h before being exposed during 48h to the virus at a multiplicity of infection of 20. Since TGF-β1 negatively controls the cell cycle of primitive HSC, 27, 28 we omitted the prestimulation step in the TGF-β1−/− group. At the end of the infection protocol, the cellular amplification was 1.5 to 2-fold in the 2 groups and the mean CFC numbers/5,000 cells plated was comparable (153 ± 73 in WT versus 122 ± 19 in TGF-β1+/−; n = 5, respectively). Transduction efficiencies varied between experiments (11% to 36%). These variations were not related to the cell genotype, but merely due to the different batches of virus used which were freshly prepared for each experiment. Given the low virus integration level and to ascertain a high chimerism in the long-term, each recipient was injected with 4 to 6 x 10^6 cells corresponding to the Lin− fraction isolated from 3 donors. With one exception due to a splenic rupture, all transplanted recipients survived longer than 6 months.

Levels of engraftment were analyzed by tracking expression of the GFP reporter gene by FACS in mature nucleated blood cells. All mice displayed GFP-fluorescence in leukocytes. However, the percentage of GFP+ cells was variable between each individuals ranging from 10% to 96% of GFP-marking at any time. These variations were not consistent with the degree of transduction at the end of the infection protocol and were not related to the cell genotype. As illustrated in Figure 1 (2 representative experiments), of 4 mice engrafted with a pool of WT cells showing an initial transduction level of 18% and 4 mice engrafted with a pool of TGF-β1−/− cells with an initial transduction level of 30%, one mouse in each group (WT12 and KO12) showed 60% and 72% GFP+ leukocytes in blood, respectively; WT9 and KO10 had about 10%, while the others displayed intermediate levels with 46% GFP+ (WT4 and KO13) or 36% GFP+ leukocytes for WT7 and KO9. Nevertheless, mice showing the highest percentage of GFP+ cells at week 6 remained relatively stable over time, while no significant increase in GFP expression was noted in animals with low expression (data not shown). To more precisely evaluate the percentage of transduced progenitor cells
over time, marrow cells were seeded in methylcellulose at sacrifice time and CFC-derived colonies were subjected to PCR with primers amplifying the viral TPO cDNA. As seen for the analysis of GFP+ leukocytes, the percentage of virus-transduced CFC in marrow was variable with high or low levels of infection detected in both groups (Table 1). However, the largest variations were observed in the TGF-β1−/− group with some animals demonstrating up to 96% of infected progenitor cells, while others were below 5%. One animal (KO7) showing no infected progenitors 4 months after the graft was discarded from the data. In some mice, the percentage of virus-infected CFC detected at week 6 or 16 was higher than immediately after infection suggesting that transduction could be more efficient in short-term, and possibly in long-term repopulating cells, than in CFC.

**TPO levels in plasma**

Baseline levels measured in plasma from normal adult wild-type mice from this colony was ≤ 1.7 ± 0.2 ng/mL (n = 10). In all transplanted mice, TPO levels were sharply increased at week 3 with values reaching up to 100 ng/mL. This elevation was sustained during 9 weeks in both groups (Figure 2). However, while values remained high in animals transplanted with WT cells over time, TPO levels progressively decreased in hosts transplanted with TGF-β1−/− donor cells.

**Hematological analysis**

In accordance with TPO elevation in circulation, platelet numbers in mice reconstituted with WT cells increased over 6 weeks achieving values 4-fold higher than normal controls (4.8 ± 0.6 x10⁶/µL versus 1.1 ± 0.2 x10⁶/µL, respectively). Thereafter, a progressive drop occurred but all mice remained thrombocytopenic with numbers 2-fold above normal. No correlation between TPO levels and platelet numbers was observed. The elevation in platelet numbers in mice engrafted with TGF-β1−/− cells was even more striking with animals maintaining values above 6 x 10⁶/µL at 4 months posttransplantation (Figure 3A). The excessive platelet production is likely related to the inhibitory effect of TGF-β1 on thrombopoiesis. Mononuclear blood cells were increased in both groups of mice (Figure 3 B) due to a striking increment in mature polymorphonuclear neutrophils in association with immature myeloid precursor cells (data not shown). However, leukocyte numbers were consistently more elevated in mice reconstituted with WT cells suggesting a stimulatory effect of TGF-β1 on granulopoiesis. Mice in both groups became progressively anemic (Figure 3 C). These data indicate that overproduction of TPO in hosts repopulated with WT or TGF-β1−/− hematopoietic cells resulted in a comparable myeloproliferative syndrome.
Chimerism of mice repopulated with TGF-β1^{-/-} transduced cells

To ascertain that hematopoietic reconstitution of wild-type hosts engrafted with TGF-β1^{-/-} cells was donor-derived in the long-term, we performed a PCR analysis for the neo gene indicative of TGF-β1 mutated allele on CFC-derived colonies from the marrow. Except for one animal (KO7), between 64% to 100% of CFC were positive for the neo gene at week 16 (Table 2). In addition, when immunocytochemistry was carried out on spleen sections with an anti-TGF-β antibody, immunolabeling showed a strong positivity in megakaryocyte and granulocyte from mice repopulated with WT cells (Figure 4 A), while no reactivity was seen in mice repopulated with TGF-β1^{-/-} hematopoietic cells (Figure 4 B). No immunostaining was observed when the anti-TGF-β antibody was omitted (Figure 4 C and D). These results demonstrate that TGF-β1^{-/-} stem cells were able to ensure a high level and long-term engraftment in wild-type irradiated hosts.

Pathological changes in tissues

Mice were euthanized at week 6 and 16 posttransplantation to examine the pathological changes. All animals displayed a splenomegaly at week 6. At week 16, spleen weights were decreased but remained 4-fold above normal in mice engrafted with WT cells, while they were slightly above normal in the TGF-β1^{-/-} group (Table 3). Irrespectively of the transplant, the marrow cellularity remained lower than in the controls at all times studied. At week 6, numbers of CFC were highly augmented in spleen and blood (20-fold and 1,000-fold, respectively) and 2-fold decreased in the marrow, but no major difference was seen between the 2 groups (Table 3). After 4 months, although spleen sizes were augmented in the WT group, the cellularity was quite low (1.28±0.65 x 10^8 cells/spleen as compared to 1.75 ±0.56 x 10^8 /spleen in animals reconstituted with cells from TGF-β1^{-/-} donors, n=3). Compared to week 6, CFC numbers were sharply decreased in spleen and blood from mice repopulated with WT cells, but the extend of decreasing was far less pronounced in TGF-β1^{-/-}-reconstituted mice (Table 3).

Histologically, spleens from animals repopulated with WT or TGF-β1^{-/-} cells appeared similar whether examinations were performed at week 6 or 16. The spleen architecture was partly preserved. The major change was a hyperplasia of the red pulp due to an extensive proliferation of heterogeneous megakaryocytes often found in clusters and numerous maturing granulocytes (Figure 5 A and 5 B). However, when silver impregnation was used to examine the degree of fibrosis, densification of the reticulin network with deposition in the vicinity of megakaryocytes was observed as early as week 6 post-graft in mice repopulated
with WT cells (data not shown). At 4 months, fibrosis was dense and severe (Figure 5 C). In sharp contrast, no densification of the reticulin network was seen in the TGF-β1−/− group during the time course (Figure 5 D). A hyperplasia of megakaryocytes and granulocytic cells was also observed on sections from the femurs (Figure 5 E and 5 F). Upon silver impregnation, myelofibrosis was prominent in mice repopulated with WT cells (Figure 5 G), while no changes in densification of reticulin was seen in the mice engrafted with TGF-β1−/− hematopoietic cells (Figure 5 H). In addition, an increase in the femoral cortical thickness and formation of new bony trabeculae extending within the bone cavity were systematically observed in the WT group (Figure 5 E and 5 G). Osteosclerosis was not seen in the mice repopulated with TGF-β1−/− cells (Figure 5 F and 5 H). In all the animals, extramedullary hematopoietic foci composed of megakaryocytes and neutrophils were observed in the liver, particularly in the sinusoidal spaces and along the portal tracts. No architectural change was noted in the kidney. However, one abnormality found in all the engrafted mice was small infiltrates of lymphocytes in the lung.

**TGF-β1 levels in plasma, platelet extracts and extracellular fluid of spleen**

Changes of TGF-β1 levels were measured in PPP during the time-course. Level of spontaneously active TGF-β1 was unsignificant whether plasma was prepared from mice reconstituted with WT or TGF-β1−/− cells. When latent TGF-β1 was activated by acidification of the samples, no increase over levels found in normal hosts could be demonstrated in mice reconstituted with TGF-β1−/− hematopoietic cells. In contrast, levels of latent TGF-β1 were 4 to 8-fold increased at week 3 posttransplantation and remained elevated during the follow-up in the mice repopulated with WT cells (Figure 6). Levels of TGF-β1 were compared in platelet extracts and extracellular fluid of spleens from normal mice and mice engrafted with WT cells (week 12 post-engraftment). In all the samples, immunoreactive TGF-β1 was at or slightly above the detection limit of the assay (62.5 picog/mL). After acidification, levels in platelet extracts were not significantly different (49.6±0.9 nanog/4x10^8 platelets versus 40.5±0.8 nanog/4x10^8 platelets, respectively; n=3 ). However, a 4-fold increase over controls could be demonstrated in extracellular fluids from WT spleens (19.1±3.7 nanog/mL versus 5.7±1.2 nanog/mL in controls; p< 0.001 ; n=3). Together, these results show that levels of latent TGF-β1 were increased in plasma and extracellular fluids of spleen from mice that developed a myelofibrosis and indicate that released of TGF-β1 by hematopoietic cells has a major impact in myelofibrosis induction.
Discussion

The critical role of TGF-β1 has been established in a variety of fibrotic disorders, but the impact of this cytokine in the development and progression of myelofibrosis has not been fully investigated. To address this issue, weexploited an experimental murine model in which overexpression of TPO by genetically modified hematopoietic cells induces severe fibrosis in hemopoietic organs. The present study compared the pathological changes induced in irradiated wild-type recipients repopulated in the long-term with TPO-overexpressing hematopoietic stem cells from homozygote mutant TGF-β1+/- or WT littermates. Since homozygote TGF-β1+/- mice die of a multifocal inflammatory syndrome at weaning time, it was not possible to perform these experiments in a background totally deficient in TGF-β1. Nevertheless, the data demonstrate the induction of a comparable myeloproliferative syndrome in hosts repopulated with TGF-β1+/- or WT marrow cells. However, while no sign of fibrosis was seen in spleen and marrow from hosts reconstituted with TGF-β1+/- donor cells (marrow chimerism > 70%), severe reticulin deposition was systematically observed in animals reconstituted with WT cells.

Previous studies on myelofibrosis induced in rodents by a chronic exposure to high TPO levels have suggested an implication of both TGF-β1 and PDGF-B in the stromal reaction. However, a key impact of PDGF-B may be ruled out since a hyperexpression of this growth factor in mice caused a myeloproliferative syndrome, but no myelofibrosis. To determine the contribution of TGF-β1, we undertook studies with TGF-β1-deficient hematopoietic stem cells. In the TGF-β1 knockout models, the frequency of birth of homozygote null pups is highly dependent on the genetic background of the mothers. In this study, marrow transplants were obtained from TGF-β1+/- pups born on a mixed Sv129 x CF-1 genetic background where a high proportion of born homozygotes develop normally until 2 weeks before they exhibit the wasting syndrome. One concern with marrow grafts originating from TGF-β1+/- donors was the possibility to transplant the acute inflammatory syndrome as previously reported. In our experiments, none of the mice repopulated with Lin- cells from TGF-β1+/- donors developed clinical symptoms of acute illness or died with exaggerated inflammation during the follow-up of 8 months. Nevertheless, all the animals transplanted with WT or TGF-β1+/- cells developed small inflammatory lesions in the lung which may be attributive to some immunological reaction induced by the mixed genetic background of these animals.

Whatever the cell genotype, high and reproducible transduction levels in hematopoietic progenitors were difficult to achieve. In preliminary experiments, the Lin- fractions from both genotypes were cocultured on the Gp+E86 ecotropic packaging cell line.
producing the MPZenTPO virus as we previously described. Surprisingly, the proviral cDNA was detected in < 5% of CFC at the end of the infection protocol, while over 80% were infected when C57Bl/6 marrow cells were tested as a control. Semi-quantitative RT-PCR analysis of the ecotropic retrovirus receptor mRNA revealed a similar expression level in Lin- cells from Sv129 x CF-1 and C57Bl/6 mice, an observation which could not explain the resistance of Sv129 x CF-1 cells to infection (data not shown). To improve transduction efficiency, we produced retroviral particles pseudotyped with the VSG glycoprotein G which can be concentrated to high titers. Accordingly to the demonstration that a short ex vivo prestimulation of primitive hematopoietic stem cells was sufficient to confer efficient gene transfer with VSV-G pseudotyped retroviruses, Lin- cells from WT donors were induced to proliferation during 24 h before being exposed to virions. The prestimulation step was omitted with the TGF-β1-/- Lin- cells since several reports indicate that autocrine TGF-β1 maintain hematopoietic stem cells in a quiescent state. Regardless of the cell genotype or the infection protocol, the percentage of transduced CFC was not significantly different between the 2 groups of mice, but never exceeded 36%. Further investigations are needed to understand the relative resistance to retrovirus infection of hematopoietic cells from Sv129 x CF-1 mice.

The comparison of the pathological changes seen in mice repopulated with TPO-overexpressing WT or TGF-β1-/- hematopoietic cells disclosed common features. All mice developed thrombocytosis, leukocytosis and greatly increased numbers of progenitors in blood and spleen. When viewed in combination, no striking differences in the myeloproliferative syndrome induced in both groups of mice were seen indicating that the repopulating and proliferating ability of stem cells from TGF-β1-/- donors was not markedly impaired. Upon histologic examination, hemopoietic tissues were invaded with an excess of megakaryocytes and granulocytes. However, while myelofibrosis was systematically observed in hemopoietic tissue from mice reconstituted with WT cells as early as week 6 posttransplantation, no reticulin deposition was seen in mice repopulated with TGF-β1-/- hematopoietic cells even after 4 months. Since hosts were wild-type animals producing TGF-β1 constitutively, these observations support the notion that TGF-β1 released by hematopoietic cells plays a prominent role in the promotion of myelofibrosis. Of note, the frequency of CFC in spleen and blood were greatly decreased in the WT group at time of myelofibrosis development. This is merely related to the difficulty to extract cells from the fibrous tissue. However, a direct inhibitory effect of TGF-β1 on primitive progenitor cells cannot be ruled out. In addition, osteosclerosis occurred after 4 months in all the mice engrafted with WT cells. This pathological process was not observed in mice repopulated...
with TGF-β1−/− cells. Although we cannot exclude that abnormal bone growth may occur at later times in these animals, it is recognized that TGF-β1 plays a pivotal role in the coupling between bone formation and resorption by stimulating osteoblasts and suppressing osteoclastogenesis. It is thus tempting to speculate that, in addition to its inductive effect on myelofibrosis, TGF-β1 may also be directly or indirectly involved in the abnormal bone growth observed in this animal model.

Measurements of TGF-β1 levels in plasma and spleen extracellular fluids from mice reconstituted with WT cells showed a 4 to 8-fold increase over normal hosts. In contrast to a previous work, no significant increment was found in platelet extracts. This may be related either to the different strategies used to induce myelofibrosis, the time of examination or the animals. However, no immunoreactive TGF-β1 was measured in the circulation even when myelofibrosis was massive. Accordingly, we were unable to identify fibrotic lesions in distant organs known to be targets to the fibrogenic effect of TGF-β1, such as the lung, liver or kidney. This indicates that critical regulatory mechanisms controlling TGF-β1 activation must take place within the hematopoietic environment at secretion sites. Cells involved in the abnormal secretion of TGF-β1 are not fully identified. Several observations postulate an important role of the megakaryocyte. Hyperplasia and disturbed differentiation of megakaryocytes are observed in human primary idiopathic myelofibrosis and in animal models of TPO hyperstimulation. In addition, on marrow biopsies, reticulin fibers are often seen in close vicinity to megakaryocytes, an observation also made in our murine model. However, other studies suggest an implication of the monocyte/macrophage. Indeed, myelofibrosis is seen in all subtypes of human myelodysplastic or chronic myeloproliferative disorders and no fibrotic process was observed in monocyte-deficient NOD-SCID mice treated with an adenovirus vector expressing the human TPO protein. Although our studies demonstrate an absence of reticulin deposition when hematopoietic cells are deficient in TGF-β1 production, we cannot exclude the possibility that some hypothetic accessory cells which might be required for myelofibrosis promotion are functionally impaired in TGF-β1−/− hematopoietic cells. The mechanisms involved in the local activation of TGF-β1 are complex and not yet understood. Nevertheless, recent studies suggest that megakaryocytes and/or monocytes might play a central role in the process of TGF-β activation in vivo. Thrombospondin-1 or integrins αvβ6, αIIbβ3 and αvβ3 are potent in vivo activators of TGF-β1. Since TGF-β1 and thrombospondin 1 are synthesized and stored within the same organelles in the megakaryocyte, it is tempting to speculate that the abnormal release of the two molecules in the microenvironment may contribute to local TGF-β1 activation. Furthermore, megakaryocytes and platelets express the integrins αIIbβ3 and αvβ3 which may
also participate to the local activation of TGF-β1. Alternatively, monocytes are sources of cytokines that are potent stimulators and activators of TGF-β1 in vivo. The availability of knockout mice for these different pathways would be useful models to dissect the mechanism(s) accounting for TGF-β1 activation in the marrow environment.

The present studies add to our understanding of the pathophysiology of myelofibrosis by demonstrating that TGF-β1 overproduction is a major contributor to the pathogenesis of this process. Although the molecular mechanisms leading to aberrant clonal proliferation of malignant hematopoietic cells in human syndromes with myelofibrosis are not fully understood, the mouse model offers an experimental assay to test new therapeutic approaches aiming to decrease TGF-β1 production and/or activation and to study the beneficial effects on the progression of myelofibrotic disorders in which no drug therapy exists.
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References


Table 1
Transduction levels in progenitor cells from hosts engrafted with WT or TGF-β1−/− virus-infected cells

<table>
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<tr>
<th>Time after transplantation</th>
<th>WT transplant mouse</th>
<th>TPO / actin</th>
<th>% positive</th>
<th>TGF-β1−/− transplant mouse</th>
<th>TPO / actin</th>
<th>% positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>week 6</td>
<td>WT 1</td>
<td>10/28</td>
<td>36%</td>
<td>KO 9</td>
<td>7/21</td>
<td>33%</td>
</tr>
<tr>
<td></td>
<td>WT 3</td>
<td>21/30</td>
<td>70%</td>
<td>KO 10</td>
<td>2/25</td>
<td>8%</td>
</tr>
<tr>
<td></td>
<td>WT 4</td>
<td>27/30</td>
<td>90%</td>
<td>KO 12</td>
<td>10/17</td>
<td>59%</td>
</tr>
<tr>
<td></td>
<td>WT 11</td>
<td>9/30</td>
<td>30%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>week 16</td>
<td>WT 2</td>
<td>7/26</td>
<td>26%</td>
<td>KO 1</td>
<td>2/28</td>
<td>7%</td>
</tr>
<tr>
<td></td>
<td>WT 5</td>
<td>5/30</td>
<td>16%</td>
<td>KO 7</td>
<td>0/30</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>WT 8</td>
<td>12/28</td>
<td>43%</td>
<td>KO 8</td>
<td>27/28</td>
<td>96%</td>
</tr>
<tr>
<td></td>
<td>WT 14</td>
<td>4/30</td>
<td>13%</td>
<td>KO 11</td>
<td>1/29</td>
<td>3%</td>
</tr>
<tr>
<td></td>
<td>KO 14</td>
<td>19/30</td>
<td>63%</td>
<td></td>
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</tr>
</tbody>
</table>

Irradiated wild-type hosts were engrafted with virus-infected marrow cells from WT or TGF-β1−/− (KO) donors. CFC-derived colonies from marrow (30 per animal) were picked from methylcellulose. Samples were analyzed by PCR with primers specific for the viral TPO gene and with actin primers to ascertain the presence of material.
Table 2
Chimerism of wild-type hosts reconstituted with marrow transplants from TGF-β1−/− donors

<table>
<thead>
<tr>
<th>Time after transplantation</th>
<th>Mouse number</th>
<th>neo/actin</th>
<th>% chimerism</th>
</tr>
</thead>
<tbody>
<tr>
<td>week 6</td>
<td>KO 9</td>
<td>21/25</td>
<td>84%</td>
</tr>
<tr>
<td></td>
<td>KO 10</td>
<td>25/28</td>
<td>89%</td>
</tr>
<tr>
<td></td>
<td>KO 12</td>
<td>17/25</td>
<td>70%</td>
</tr>
<tr>
<td>week 16</td>
<td>KO 1</td>
<td>25/39</td>
<td>64%</td>
</tr>
<tr>
<td></td>
<td>KO 7</td>
<td>5/22</td>
<td>22%</td>
</tr>
<tr>
<td></td>
<td>KO 8</td>
<td>21/28</td>
<td>75%</td>
</tr>
<tr>
<td></td>
<td>KO 14</td>
<td>30/30</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>KO 11</td>
<td>30/30</td>
<td>100%</td>
</tr>
</tbody>
</table>

Irradiated wild-type hosts were engrafted with virus-infected marrow cells from TGF-β1−/− donors (KO). Marrow CFC-derived colonies (30 per recipient animal) were picked and samples were analyzed by PCR with primers specific for the neo gene indicative of the mutated TGF-β1 allele (TGF-β1−/− donor cells) and with actin primers to ascertain the presence of material.
Table 3
Analysis of mice reconstituted with WT and TGF-β1−/− marrow cells infected with the MIGR-TPO virus.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>items</th>
<th>Wild-type control</th>
<th>week 6 posttransplantation</th>
<th>week 16 posttransplantation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow</td>
<td>cell/femur (x10⁶)</td>
<td>29.2 ± 3.3</td>
<td>16.2 ± 2.7</td>
<td>16.5 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>CFC/femur (x 10³)</td>
<td>86.5 ± 8.3</td>
<td>49.2 ± 7.4</td>
<td>48.6 ± 9.1</td>
</tr>
<tr>
<td>Spleen</td>
<td>weight (mg)</td>
<td>126 ± 20</td>
<td>595 ± 88</td>
<td>1,025 ± 225</td>
</tr>
<tr>
<td></td>
<td>CFC/10⁶ cells</td>
<td>88 ± 22</td>
<td>1,280 ± 123</td>
<td>1,845 ± 250</td>
</tr>
<tr>
<td>Blood</td>
<td>Nucleated cells/ml (x 10⁵)</td>
<td>9.6 ± 0.7</td>
<td>70.5 ± 30.6</td>
<td>67.5 ± 31.5</td>
</tr>
<tr>
<td></td>
<td>CFC/ml (x 10²)</td>
<td>&lt; 1</td>
<td>1,100 ± 260</td>
<td>590 ± 100</td>
</tr>
</tbody>
</table>

Values are mean ± SD of data from 3 controls and 3 mice in each group. Progenitor cells numbers (CFC) were calculated from the number of colonies obtained from 1 x 10⁵ cells grown in semi solid medium. * Statistically not significant. P< 0.1.
Figure legends

**Figure 1**: Flow cytometry profile showing green fluorescent protein (GFP) positive cells. at the end of the infection protocol (A) and in peripheral blood leukocytes (B) of individual mouse engrafted with virus-infected cells at week 6 posttransplantation. WT transplant (left panels) or TGF-β1<sup>−/−</sup> transplants (right panels). Percent of positive cells is given above the corresponding profile.

**Figure 2**: TPO quantification in plasma. Each point is the mean ± SD of 12 to 14 animals, with the exception that only 6 TGF-β1<sup>−/−</sup> animals were analyzed at week 16. Circles, TGF-β1<sup>+/+</sup> (WT) transplant; squares, TGF-β1<sup>−/−</sup> (-/-) transplant. Results of statistical analysis with the two-tailed Student’s t-test. WT versus TGF-β1<sup>−/−</sup>. * P < 0.05 and * * P < 0.001.

**Figure 3**: Blood parameters. Evolution in platelet numbers (A), leukocyte numbers (B) and hematocrit (C) in mice transplanted with WT (circles) or TGF-β1<sup>−/−</sup> (squares) virus-infected hematopoietic cells. Each point represents the mean ± SD of 12 to 14 animals, with the exception that only 6 TGF-β1<sup>−/−</sup> animals were analyzed at wk 16. Results of statistical analysis with the two-tailed Student’s t-test. WT versus TGF-β1<sup>−/−</sup>. * P < 0.05 and * * P < 0.001.

**Figure 4**: TGF-β expression in spleen section from mice engrafted with WT or TGF-β1<sup>−/−</sup> virus-infected hematopoietic cells. Immunocytochemistry using a TGF-β specific antibody (A and B). Immunostaining specificity was checked by omitting the primary antibody (C and D). Representative sections are shown at 3 months posttransplantation. (A) mouse engrafted with WT cells showing strong positivity in MK and granulocytes. (B) mouse engrafted with TGF-β1<sup>−/−</sup> cells showing no immunoreactivity. (C and D) No immunostaining was revealed when the primary antibody was omitted. Original magnification (x600).

**Figure 5**: Histological sections of spleen and femur from mice engrafted with WT or TGF-β1<sup>−/−</sup> virus-infected hematopoietic cells. Three mice in each group (WT5, WT8, WT14 and KO8, KO11, KO14) were killed 4 months after transplantation. Representative sections are shown. Spleen sections stained by hematoxylin/eosin from (A) WT5 engrafted with TGF-β1<sup>+/+</sup> cells and (B) KO14 engrafted with TGF-β1<sup>−/−</sup> cells showing the hyperplasia of megakaryocytes and granulocytes. Silver staining revealed massive fibrosis in spleen sections from WT5 (C), while no significant reticulin deposition was detected in KO14 (D). Longitudinal femur sections stained by H&E from WT5 (E) showing the growth of new bone trabeculae within the marrow cavity and from KO14 (F) where no bone growth was detected.
Silver staining of a femur section from WT5 (G) showing myelofibrosis and from KO14 (H) showing no deposition of reticulin fibers. Original magnifications: (A, B, C and D) x 400; (E, F, G and H) x 250

**Figure 6: TGF-β1 quantification in plasma.** Bars indicate TGF-β1 levels after acidification of the samples: closed bars, levels in mice engrafted with TGF-β1+/+ hematopoietic cells; open bars, levels in hosts engrafted with TGF-β1−/− hematopoietic cells; hatched bar, constitutive level in wild-type adult hosts. No spontaneously active TGF-β1 was detected prior to acidification of the samples. Results of statistical analysis with the two-tailed Student’s t-test. WT versus TGF-β1−/−. * P < 0.05 and ** P < 0.001.
Figure 1

A  WT         TGF β−/−

B  WT 12       KO 12
  60%          72%

WT 4         KO 13
  46%          46%

WT 7         KO 9
  36%          34%

WT 9         KO 10
  11%          12%
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Prominent role of TGF-β1 in thrombopoietin-induced myelofibrosis in mice

Hedia Chagraoui, Emiko Komura, Micheline Tulliez, Stephane Giraudier, William Vainchenker and Francoise Wendling

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