博士学位論文

骨髄異形成患者の巨核球様細胞に対する、ルストロンボパグの効率的な増殖と分化の促進

近畿大学大学院
医学研究科医学系専攻

佐　野　圭　吾

Doctoral Dissertation

Lusutrombopag effectively promotes the growth and differentiation of megakaryocytic cells from patients with myelodysplastic syndrome

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Lusutrombopag effectively promotes the growth and differentiation of megakaryocytic cells from patients with myelodysplastic syndrome

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同　意　書

令和3 年 11 月 10 日

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課博 ・ 論博

同　意　書

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Abstract:

Background

Thrombopoietin (TPO) and its receptor, c-Mpl are essential for megakaryopoiesis. Two TPO receptor agonists (TPO-RAs), Eltrombopag (ETP) and Romiplostim (Rom), which also activate c-Mpl, thereby transmitting signals to downstream molecules, are currently used for immune thrombocytopenia purpura and aplastic anemia. Furthermore, recent clinical trials demonstrated that ETP and Rom would be useful for Myelodysplastic syndromes (MDS) patients with thrombocytopenia. Lusutrombopag (LTP) is a novel TPO-RA, which is approved for thrombocytopenia associated with liver diseases. In this study, we examined the effects of LTP on the growth and differentiation of MDS $CD34^+$ cells as a preclinical study.

Method

Effects of LTP on the proliferation, differentiation, and long-term self-renewal activities of bone marrow $CD34^+$ cells from MDS patients were assessed with liquid cultures, CFU-megakaryocyte assays, and serial replating assays, respectively. We also analyzed the effects of LTP on cytokine secretion from cultured MDS $CD34^+$ cells with a cytokine antibody array.

Results

LTP effectively promotes the growth and differentiation of MDS $CD34^+$ cells with comparable efficacy to that exerted on $CD34^+$ cells from healthy controls. The megakaryopoietic potential of LTP was comparable to that exerted by ETP and TPO. LTP did not stimulate growth of $CD34^+$ MDS/AML blasts, a concern that has been associated with ETP and Rom. Both ETP and TPO induced higher levels of FGF7 than LTP from $CD34^+$ MDS cells.

Conclusion

Although LTP and ETP revealed some different biologic activities, LTP would be as effective as ETP in promoting the growth and megakaryocytic differentiation of MDS $CD34^+$ cells.

Keywords:

lusutrombopag; thrombopoietin receptor; hematopoiesis; megakaryopoiesis; myelodysplastic syndrome

1. Introduction

Thrombopoietin (TPO) is an essential factor for megakaryopoiesis and platelet production. Upon binding to its cognate receptor (c-Mpl), TPO evokes dimerization of the c-Mpl protein and transmits growth and survival signals to downstream molecules *via* the tyrosine kinase JAK2 pathway[1,2]. Based on its stimulatory activities on megakaryopoiesis and platelet production *in vitro* and *in vivo*, a number of clinical trials were conducted for patients with thrombocytopenia (such as those after chemotherapy and with immune thrombocytopenic purpura [ITP]), using recombinant human TPO (rhTPO) or pegylated and modified rhTPO. Although TPO showed some activities in increasing platelet counts in these studies, a small part of participants paradoxically experienced thrombocytopenia due to the appearance of neutralizing antibodies (Abs) against endogenous TPO. Thus, clinical trials using rhTPO were discontinued thereafter[3,4].

As an alternative strategy to increase platelet counts, thrombopoietin receptor agonists (TPO-RAs), such as Romiplostim (Rom) and Eltrombopag (ETP) have been developed[5-7]. After confirmation of the utility and safety in clinical trials, Rom and ETP were approved for ITP and thrombocytopenia associated with liver diseases in several countries.

Lusutrombopag (S-888711) (LTP) is a nonpeptide small molecule that acts as a novel TPO-RA like Rom and ETP. At present, LTP is approved for thrombocytopenia associated with liver diseases scheduled to undergo elective invasive procedures[6-8].

The receptor c-Mpl is also expressed on immature hematopoietic cells including hematopoietic stem cells (HSCs) and progenitor cells as well as on megakaryocytes (Mks)[1,2,9]. Later studies using HSCs from c-Mpl knockout mice revealed that the TPO/c-Mpl system is essential for the maintenance of HSCs[10-13]. From these results, it was speculated that, in addition to the lineage-restricted function (*i.e.*, promotion of megakaryopoiesis and platelet production), TPO and TPO-RAs might have a function to increase HSCs. In fact, several clinical studies demonstrated that ETP and Rom alone or combined with immunosuppressive therapy improved hematopoiesis in all lineages in patients with aplastic anemia (AA)[14], and as a result these agents were approved for their clinical use in patients with AA in several countries. Meanwhile, c-Mpl is also expressed on acute myeloid leukemia (AML) cells, thus because a considerable proportion of AA patients have gene mutations that are common with AML and myelodysplastic syndrome (MDS)[15], there is a concern that TPO-RAs might enhance the growth of HSCs with gene mutations in AA patients and thereby promote disease progression from AA to AML[16].

MDS is a clonal disorder caused by genetic and/or chromosomal aberrations in HSCs. Bone marrow (BM) insufficiency due to ineffective hematopoiesis in BM leads to cytopenia in one or more lineages (that is, anemia, thrombocytopenia, and/or neutropenia) in low-risk MDS[17-21]. Meanwhile, increased proliferation of immature myeloid cells (myeloblasts) preceding acute myeloid leukemia is a major disease characteristic in high-risk MDS[18,20,22]. Although allogeneic hematopoietic stem cell transplantation (allo HSCT) is the only therapeutic strategy to cure MDS, this therapy is often accompanied by life-threatening complications such as infections, graft failure, and acute and chronic graft-versus-host diseases. So, at present, allo HSCT isn't recommended for low-risk MDS, however, because there is no key drug capable of dramatically improving cytopenias or prolonging survival of low-risk MDS patients, only red blood cell transfusions are conducted for severe anemia as best supportive care[23,24]. Also, patients with severe thrombocytopenia often undergo repeated platelet transfusions in order to prevent lethal bleeding such as cerebral hemorrhage and gastrointestinal bleeding. As compared with red blood cell transfusion, the interval of platelet transfusions is rather short because the life span of platelets is about only 1 week[18]. Thus, a new therapeutic strategy for thrombocytopenia is needed in patients with MDS.

Thus, clinical trials using either ETP or Rom for low-risk MDS have been conducted. As a result, both TPO-RAs were effective for thrombocytopenia in terms of increasing platelet count and reducing bleeding events in low-risk MDS patients, which was not accompanied by an apparent increase of progression to AML[25].

Based on these results, we speculated that LTP might be useful to improve thrombocytopenia in patients with MDS as well as ETP and Rom. So, in this study, we examined the effects of LTP on the growth, differentiation, and survival of $CD34^+$ hematopoietic stem/progenitor cells (HSC/HPCs) isolated from MDS patients as a preclinical study. Our data demonstrated that LTP supports megakaryopoiesis from $CD34^+$ cells as efficiently as ETP and rhTPO. In addition, we found that LTP and ETP induce a different pattern of cytokine secretion from BM mononuclear cells (MNCs).

2. Experimental Section

Reagents

LTP and ETP were provided by Shionogi & Co., Ltd. (Osaka, Japan). These agents were dissolved in double distilled (dd) H_2O at different concentrations (0.1 mM, 1 mM and 10 mM) and stored in 4℃ until use. Freezing storage medium (CELLBANKER®2) was purchased from ZENOAQ RESOURCE (Fukushima, Japan). StemSpan™ Serum-Free Expansion Medium (SFEM), was acquired from STEMCELL Technologies

(Vancouver, BC, Canada). Methylcellulose medium (Methocult H4434) and MegaCult™-C Complete kit were acquired from STEMCELL Technologies. Recombinant human (rh) Interleukin-3 (IL-3), IL-6, IL-11, TPO, stem cell factor (SCF), and Flt3 ligand (FLT3L) were purchased from Peprotech (Rocky Hill, NJ). CuSO4 was purchased from Merck (Darmstadt, Germany).

Patients' samples

This study was planned and conducted in accordance with the Declaration of Helsinki with the permission from the Kindai university ethical committee (Approved ID: 26-087). BM samples were obtained from 13 MDS patients, 5 AML patients (3 patients with AML with MRC and 2 patients with AML M6 according to the classification of WHO 2016[26]), and one healthy donor after written informed consent was given. BM MNCs were separated by Ficoll™ Paque Plus (Cytiva, Tokyo, Japan), suspended in CELLBANKER®2, and cryopreserved until use. The characteristics of the 18 cases analyzed are summarized in Table 1.

Table 1. Characteristics of the MDS/AML patients at diagnosis evaluated in this study.

Case	Age	Sex	diagnosis	blast	karyotype	WT1
1	73	male	MPN-U	2.3	46,XY	<50
2	62	female	RCMD	4.5	46XX	660
3	77	male	AML(M6)	5.2/25.6	45,XY,del(5)(q?),-17,-18,dic(20;21)(q11.2q13),+mar1,+mar2 [6/20]	-
4	64	female	sMDS overt	26.2	45,XX,-7	77000
5	54	female	sMDS(RCMD)	7.3	45,XX,t(1;3)(p13p23),dic(5;12)(q11.2;p11.2),i(22)(q10) [6/20]	1700
6	70	male	RAEB-1	6.4	46,XY,+1,der(1;7)(q10;p10),del(20)(q11.2q13.3) [19/20]	880
7	52	male	RCMD	1	46,XY,der2t(1;2)(q12;q37)[2]/46,XY[18]	-
8	58	male	AML(M6)	6.3	47,XY,+8[3]/47,idem,-7,del(12)(p?),+r[17]	1300
9	57	male	RCMD	3.5	46,XY,+1,der(1;7)(q10;p10)[11]	1000
10	73	male	RAEB-1	7.3	45,XY,-7[16]/47,idem,del(12)(p?)[4]	290
11	75	female	RCMD	1.9	46,XX,t(5;10)(q13;q24)	<50
12	61	male	RCMD	3.7	47,XY,+18[1]	<50
13	70	male	AML/MRC	40.8	46,XY	17000
14	80	male	AML/MRC	28.2	45,Xyadd(12)(p11.2),-16[8]/44,idem,add(1)(q32),add(3)(p21),-7,?t(11;13)(p15;q12),-17,-17,+ma1,+mar2[4]	42000
15	72	male	AML/MRC	56.6	46,XY,add(6),-7,add(8)(p.11.2),add(14)(q24),add(21)(p11.2),+mar[2]	9000
16	84	male	RCMD	0.7	46,XY	—
17	72	male	RAEB-Ⅱ or CMML-2	13.8	46,XY	230
18	86	male	RAEB-Ⅰ	6.8	45,XY,-2,add(5)(q11.2),-7,+8,-10,add(11)(p11.2),der(12;21)t(12;?)(p11.2;?)t(21;x)(q22;?)x2,-17,add(20)(q11.2), +21, +mar1, +mar2	15000

Assessing the proliferation of CD34$^+$ cells

CD34$^+$ cells were isolated from fresh or cryopreserved BM MNCs with CD34 microbeads (CD34 MicroBead Kit, Bergisch Gladbach, Germany) according to the manufacturer's protocol. Isolated CD34$^+$ cells were suspended at 1.0×10^4 cells/ml in triplicate and cultured for 7 or 14 days in SFEM medium supplemented with the following cytokines: 10 ng/ml rhIL-3, 25 ng rhIL-6, 50 ng/ml rhSCF and rhFlt3L. The following agents were added as indicated: 100 ng/ml rhTPO, 3 μM or 10 μM ETP with 5.0 μM CuSO4 (indicated as ETP), or 3 μM or 10 μM LTP. Cell proliferation was assessed using the Cell Titer-Glo® Luminescent Cell Viability assay (Promega, Madison, WI) from the

intensity of luminescence with an Envision plate reader (Wallac, 1420 ARVO MX-2, Turku, Finland) according to the manufacturer's instruction.

Flow cytometry analysis

Cultured cells were harvested and stained with the following Abs or dye: anti-human CD34 APC Ab, anti-human CD41 FITC Ab, anti-human CD45 APC/cy7 Ab, and 7-AAD. Then, the cells were analyzed with a FACS Canto^{TM}II flow cytometry instrument (BD Biosciences, San Diego, CA).

Colony forming assay and replating assay

To assess the effects of TPO-RAs on the growth and differentiation of $CD34^+$ cells, we used colony forming assays. In brief, $CD34^+$ cells were suspended in methylcellulose medium at a density 1,000 cells/ml and cultured with the following factor: $CuSO_4$, rhTPO, ETP, or LTP at various concentrations in duplicate. After 14-day cultures, colony forming units (CFU) were inspected and counted. We also performed serial replating assays as previously described[27]. After 12-14-day cultures, colony-forming cells were harvested and suspended as a single cell. These cells were cultured in methylcellulose medium at the same cell density for another 14 days under the same condition. These procedures were repeated until no colonies formed.

Megakaryocytic colony forming assay

To examine the effects of LTP and ETP on the growth and megakaryocytic differentiation of MDS $CD34^+$ cells, we used a megakaryocyte CFU (CFU-Mk) assay. $CD34^+$ cells obtained from MDS cases were suspended at a cell density of 1.0×10^5 cells/ml in MegaCult^{TM}-C kit without cytokines medium (STEMCELL Technologies, Vancouver, Canada) supplemented with 40 μg/ml of low-density lipoprotein, 10 ng/ml IL-6, and 10 ng/ml IL-3 with or without LTP, ETP, or TPO. After 14-day cultures, the developed colonies were stained using an anti-human GPIIb/III (CD41a) Ab and observed under a microscope (CX41, OLYMPUS, Tokyo, Japan) equipped with a digital camera (DS-Ril, Nikon). Morphology of colony-forming cells was analyzed by the May-Grunwald-Giemsa staining on cytospin preparations. Colonies were classified into four types of colonies by their size and cell types, including small, medium, large or mixed colonies following the manufacturer's recommended protocol.

Cytokine secreting assay

$CD34^+$ cells from 5 MDS patients (Cases #1-#5) were suspended in megakaryopoietic medium (StemSpan™ SFEM medium supplemented with 10 ng/ml rhIL-6, 10 ng/ml rhIL-11) at a density 1.0×10^5 cells/ml in duplicate. Cells were cultured for 7 days in the presence or the absence of the following reagent: 100 ng/ml rhTPO, 10 μM ETP and 5 μM CuSO4, or 10μM LTP. Cell culture supernatants were collected and the concentrations of soluble factors released from the cultured cells were assessed using a protein array (Human Growth Factor Antibody Array C1, RayBio®, Norcross, GA), following the manufacturer' protocol. The intensities of signals were quantified by a densitometry in duplicate.

Data analysis

Data were analyzed using the 'EZR' software package[28]. When appropriate, one-way analysis of variance (one-way ANOVA) and repeated measures analysis of variance (repeated measures ANOVA) were performed. Error bars indicate the standard deviation (SD) of the mean. Differences were analyzed by the Tukey-Kramer method or the Dunnett method. P-values less than 0.05 were considered statistically significant.

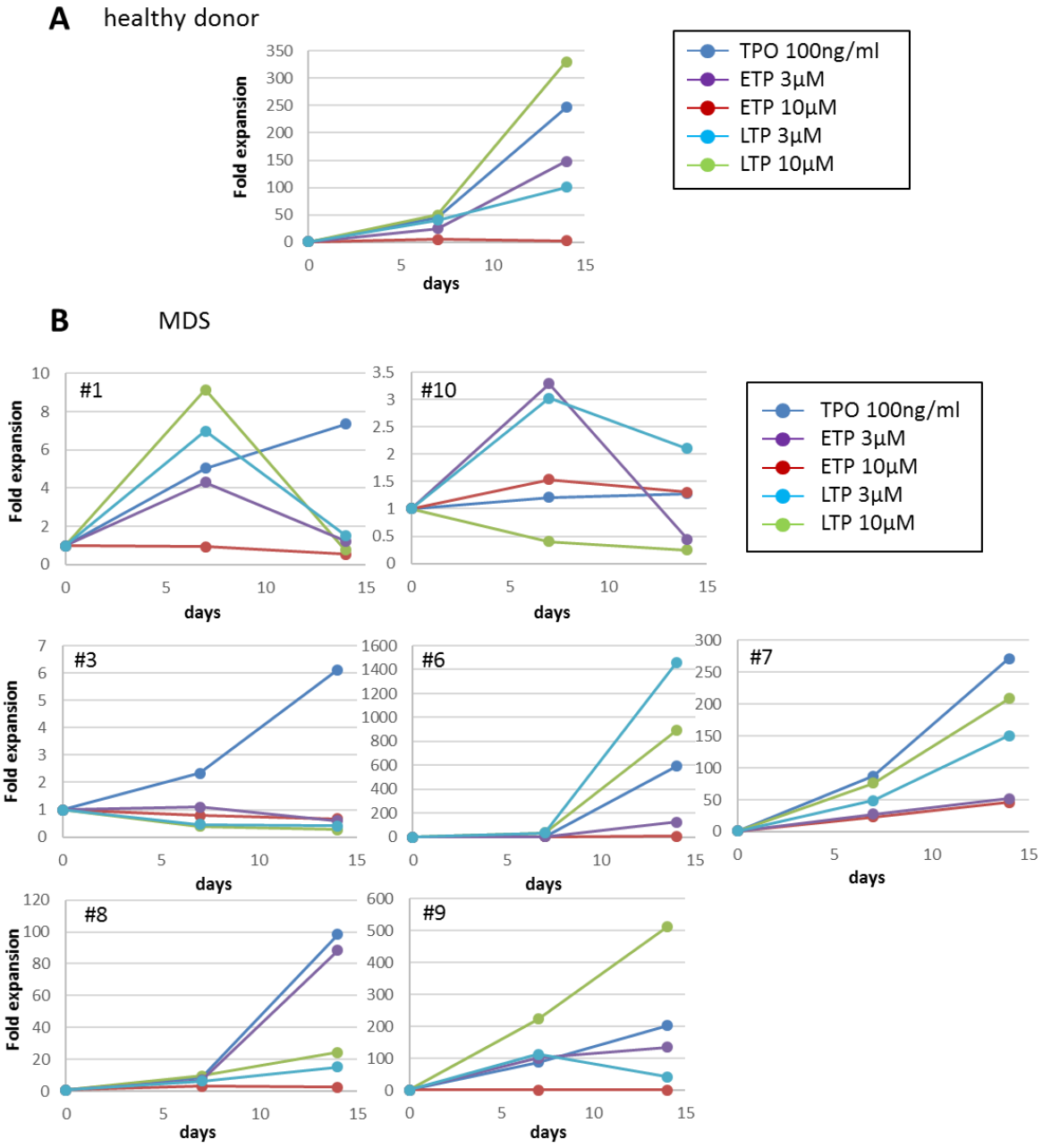
3. Results

Effects of TPO-RAs and rhTPO on the proliferation of BM $CD34^+$ cells

At first, we assessed the effects of ETP (3 μM and 10 μM), LTP (3 μM and 10 μM), and rhTPO (100 ng/ml) on the proliferation of BM $CD34^+$ cells from 7 MDS cases (cases #1, #3, #6, #7, #8, #9, #10) and one healthy donor with liquid cultures. $CD34^+$ cells were cultured with these TPO-RAs at pharmacological concentrations observed in patients receiving their oral administration[29]. In addition, $CuSO_4$ was added for stabilizing *in vitro* activity of ETP[30]. In the presence of background growth factors (rhIL-3, rhIL-6, rhSCF and rhFlt3L), normal $CD34^+$ cells vigorously proliferated in response to 3 μM, 10 μM LTP, 3 μM ETP, and rhTPO but not to 10 μM ETP. $CD34^+$ cells from 2 cases (cases #1 and #10) didn't persistently increase up to day14 in response to LTP, ETP, or rhTPO, except for case #1 to rhTPO. On the other hand, those from MDS cases showed various patterns of responses to TPO-RAs and rhTPO (Fig. 1B). $CD34^+$ cells from MDS case #3 increased only in response to rhTPO. In contrast, $CD34^+$ cells from MDS cases #6, #7, #8, and #9 increased in response to 3 μM, 10 μM LTP, 3 μM ETP as well as to rhTPO. The most effective CD34+ cell activation stimuli in these assays was 3 μM LTP for MDS case #6, rhTPO for case #7, rhTPO and 3 μM ETP for case #8, and 10 μM LTP for case #9, respectively. However, as observed in the culture of normal $CD34^+$ cells, none of MDS samples, except for case #7, proliferated in the presence of 10 μM ETP. These

results indicate that 3 μM/10 μM LTP and 3 μM ETP but not 10 μM ETP would promote the growth of MDS CD34^{+} cells almost as effectively as rhTPO.

Fig. 1

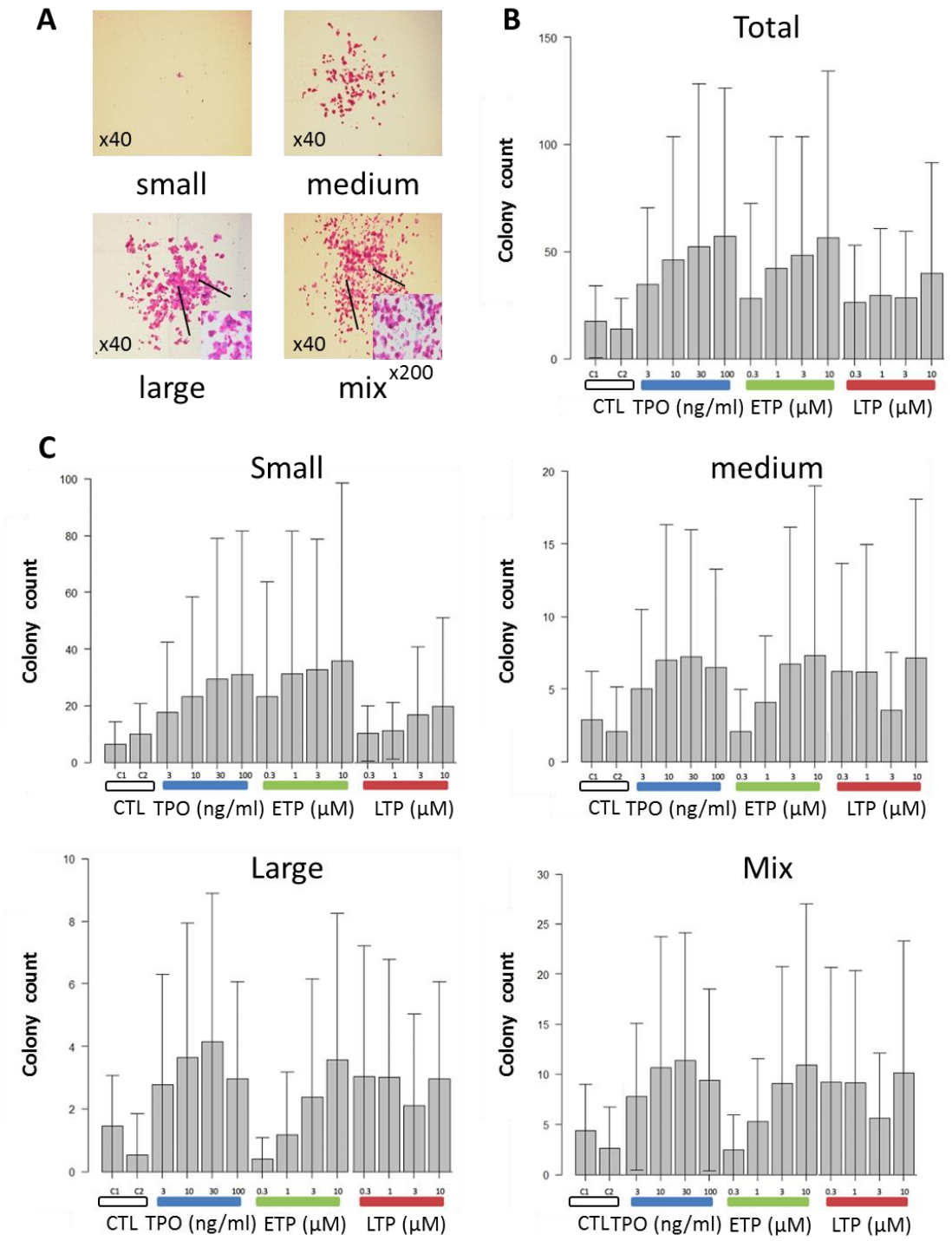


Effects of TPO-RAs and rhTPO on the megakaryocytic growth and differentiation of MDS CD34+ cells

Next, we analyzed the effects of LTP and ETP on the megakaryocytic growth and differentiation of BM $CD34^+$ cells isolated from 5 MDS cases (cases #1, #2, #5, #6, and #7) with CFU-Mk assays, using rhTPO as a reference. After 14-day cultures, we identified Mk colonies with an anti-human CD41a Ab and classified them based on their sizes into a small colony (containing 3-20 cells: likely derived from a mature Mk progenitor cell), a medium colony (containing 21-50 cells formed from an immature Mk progenitor cell), a large colony (>50 cells formed from the most immature Mk progenitor cell), and a mixed colony composed of both Mks and non-Mks (Fig. 2A). When compared with control cultures (CTL, C1 and C2), LTP, ETP, and rhTPO tended to increase total Mk colony numbers dose-dependently. However, there was no statistically significant difference between any concentrations of the tested agents due to the big case-to-case variations (Fig. 2B). Similar results were observed when we counted small, medium, and large Mk colonies and mixed colonies (Fig. 2C). These results together with those obtained from liquid cultures, suggested that LTP would promote the megakaryocytic growth from $CD34^+$ cells regardless of their progeny as efficiently as ETP and rhTPO.

We also analyzed the morphology of colony-forming cells by the May-Grünwald-Giemsa staining on cytospin preparations. As shown in Fig 2A, both LTP and ETP yielded immature and mature Mks at various maturation stages. Importantly, a considerable growth of megakaryoblasts corresponding to MDS/AML blasts was not observed in the total cell populations. These results suggest that both LTP and ETP are capable of inducing megakaryocytic differentiation of MDS CD34+ cells without promoting the proliferation of MDS/AML blasts.

Fig.2



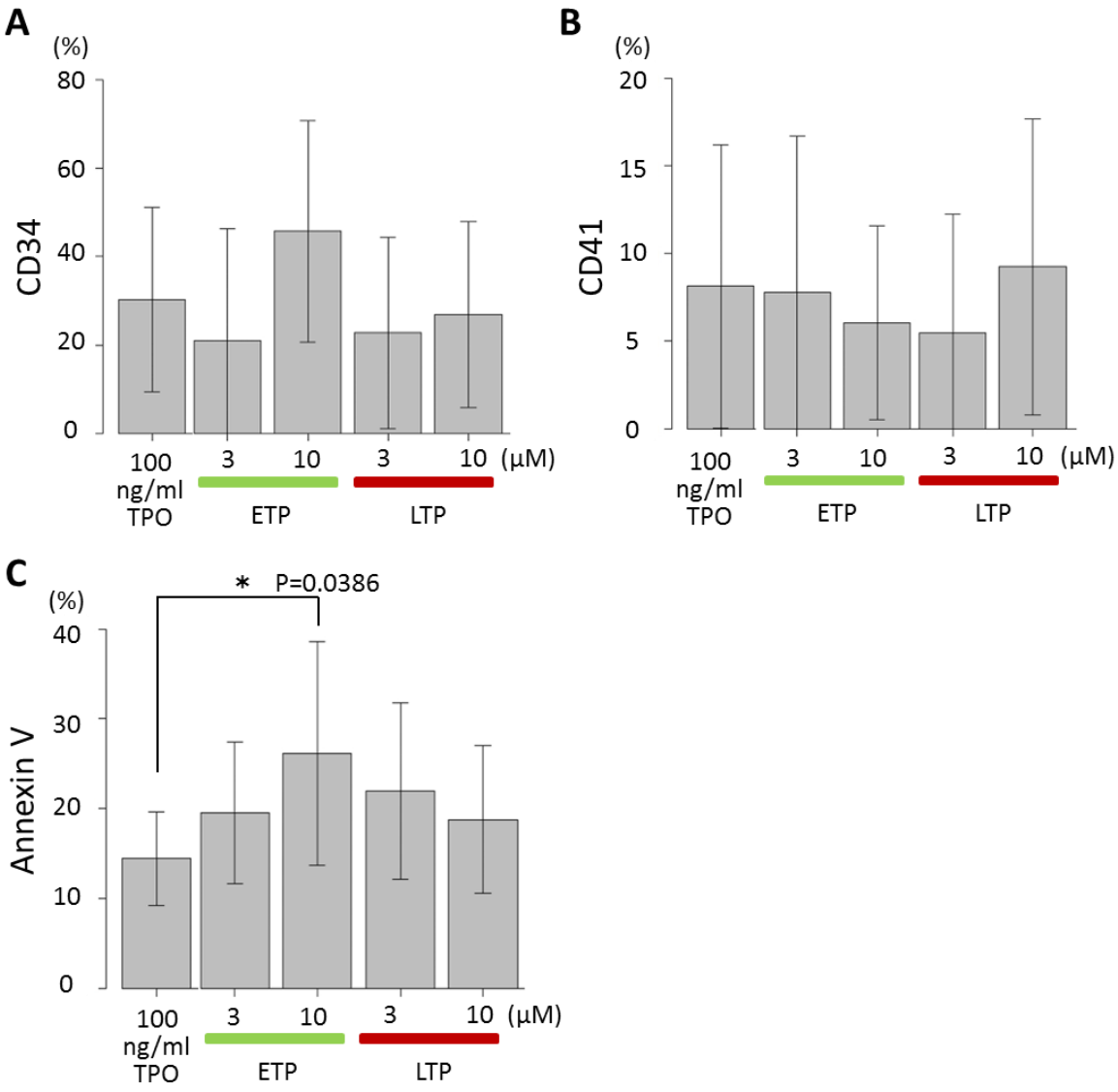
Effects of TPO-RAs and rhTPO on megakaryocytic differentiation and survival of BM CD34^{+} cells

To confirm that LTP and ETP don't increase immature MDS/AML blasts, we also analyzed the changes in the proportion of $CD34^+$ cells with liquid cultures, using $CD34^+$ cells from 6 MDS cases (cases #1, #2, #4, #5, #6, and #9). Because $CD34^+$ cells were enriched from BM MNCs to high purity using cells sorting, the proportions of $CD34^+$ cells were nearly 100% before the cultures in all samples (data not shown). As shown in Fig 3A, the proportions of $CD34^+$ cells considerably decreased in all 7-day cultures: with TPO 28.0±20.3%, 3μM ETP 20.8±22.5%, 10 μM ETP 44.1±22.9%, 3 μM LTP 22.7±19.4%, and 10 μM LTP 24.3±19.6% (without a significant difference among the cultures). These results again suggested that neither LTP nor ETP would increase $CD34^+$ MDS/AML blasts.

In the previous experiments, 10 μM ETP didn't support the growth of normal and MDS $CD34^+$ cells. Because the growth capacity of hematopoietic cells is impaired along with their differentiation, we investigated the effects of 10 μM ETP on megakaryocytic differentiation of $CD34^+$ cells. For this purpose, we analyzed the proportions of $CD41^+$ cells by flow cytometry after 7-day cultures. The proportions of $CD41^+$ cells were as follows: with TPO 7.6±9.3%, 3 μM ETP 7.4±8.8%, 10 μM ETP 6.7±5.4%, 3 μM LTP 5.9±7.1%, 10 μM LTP 9.4±7.8%, without a significant difference (Fig. 3B).

Because the growth inhibition caused by 10 μM ETP appeared to be not related with an enhanced differentiation of $CD34^+$ cells, we next examined the effects of these agents on cell survival. We stained the cells with Annexin V- and 7-AAD. 7-AAD was utilized to exclude dead cells from the analysis. As shown in Fig. 3C, the proportion of Annexin V-positive apoptotic cells was significantly higher after cell exposure to 10 μM ETP compared to cultures treated with rhTPO (10 μM ETP 26.2±13.6% *vs*. rhTPO 14.3±5.1%, p=0.0386), but without a significant difference from the other cultures (3 μM ETP 20.9±7.2%, 3 μM LTP 23.1±10.4%, and 10 μM LTP 18.9±8.4%), likely in part due to the large case-to-case variations. These results suggest that LTP does not impair the viability of $CD34^+$ MDS cells at both low and high doses, while high dose ETP might enhance apoptosis in normal $CD34^+$ and $CD34^+$ MDS cells.

Fig.3

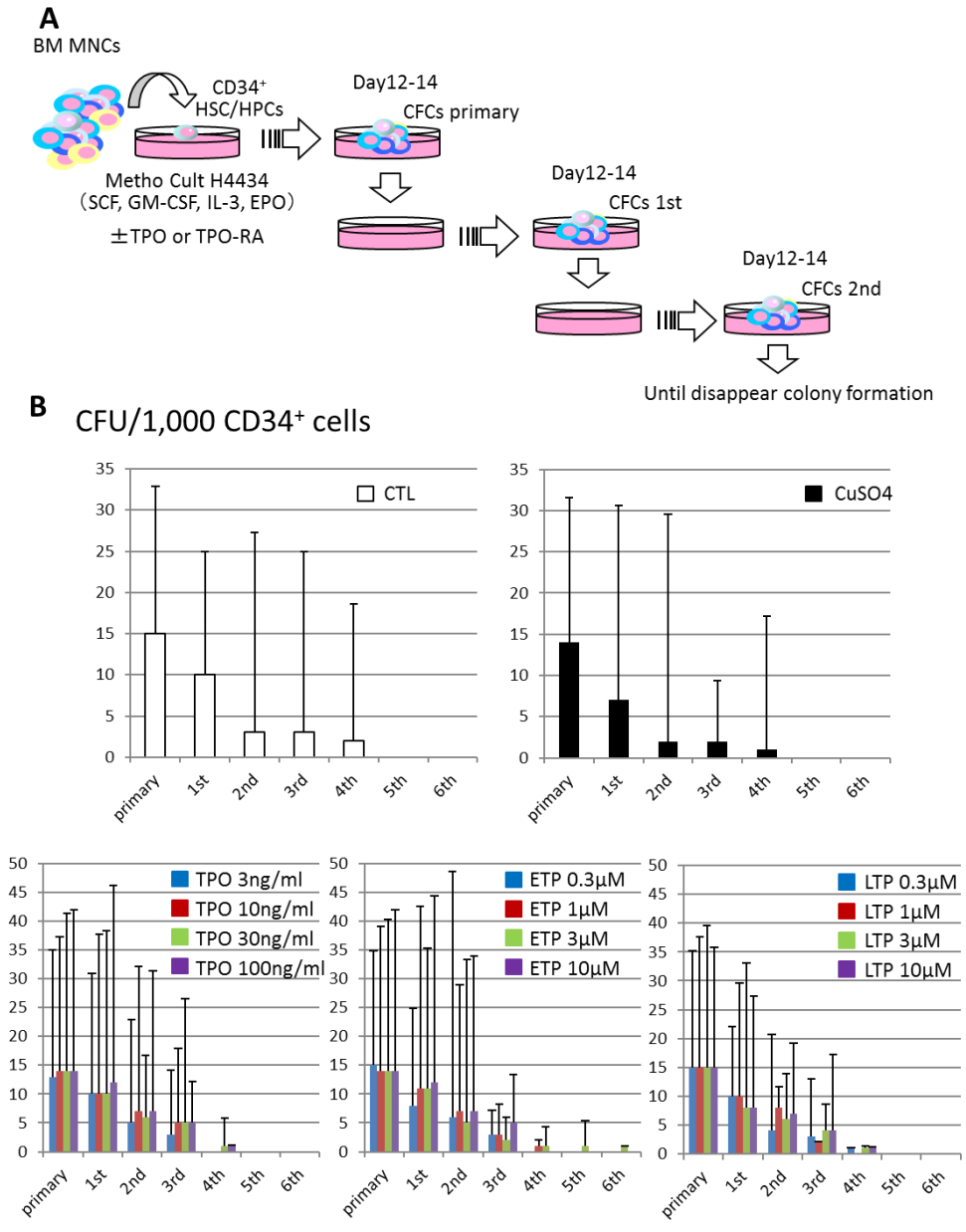


Effects of LTP and ETP on self-renewal of MDS CD34⁺ cells

We next examined the effects of LTP and ETP on long-term self-renewal activities of CD34⁺ cells from 18 MDS/AML cases with serial replating assays with rhTPO as a reference (Fig. 4A). In these experiments, we found prominent interindividual variability in the capacities to generate colonies under the control cultures and those with CuSO4 alone (Fig. 4B). Although we did not observe statistical significant differences, likely due to the limited number of samples, the colony-forming activities of samples analyzed appear to be somewhat influenced by the disease characteristics (a moderate increase of colony-forming cells in CD34+ cells derived from high-risk MDS and AML compared

with low-risk MDS was observed). Of note, none of the samples assessed was capable of replating more than 4 times under these conditions, which was the case for cultures stimulated with LTP, ETP, and rhTPO regardless of the concentrations utilized, suggesting that these reagents do not increase the self-renewal activities of MDS/AML $CD34^+$ cells (Fig. 4B). On the other hand, we observed that while ETP at 10 μM did not affect the colony-forming activities or self-renewal activities of $CD34^+$ cells, it consistently impaired the growth of normal and MDS $CD34^+$ cells (Fig. 1A, and 1B)

Fig.4



Effects of LTP and LTP on cytokine secretion from CD34$^+$ cells from MDS cases

To further characterize the effects of TPO-RAs on hematopoiesis, we analyzed cytokine secretion from hematopoietic cells stimulated with TPO-RAs with a protein array, in which we focused on cytokines implicated in the development of myelofibrosis. CD34$^+$ cells from 5 MDS cases (cases #1-#5) were incubated with 100 ng/ml TPO, 10 μM ETP, or 10 μM LTP for 7 days. Then, the cell culture supernatants were subjected to a protein array and the intensities of signals were quantified by densitometry in duplicate. The mean values of 5 MDS samples are indicated in Table 2. While rhTPO tended to reduce the secretion of some cytokines such as epidermal growth factor (EGF), heparin-binding EGF-like growth factor (HB-EGF), and vascular endothelial growth factor (VEGF) (indicated as blue boxes) compared with the control culture, it did not increase the secretion of any of cytokines evaluated in this array. In contrast, both ETP and LTP tended to increase the secretion of basic fibroblast growth factor (bFGF) and EGF compared with rhTPO and the control culture (indicated as red boxes). It was of interest that ETP and LTP tended to conversely influence the secretion of FGF-7: that is, FGF-7 secretion was stimulated by ETP but reduced by LTP. On the other hand, none of the reagents (rhTPO, ETP, and LTP) directly increased the levels of transforming growth factor-β1 (TGF-β), which is considered as a key regulator of myelofibrosis.

Table.2 Cytokine secretion from CD34$^+$ cells from 5 MDS cases after the cultures with control, 100 ng/ml rhTPO, 10μM ETP, or 10μM LTP (indicated as mean).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Pos	Pos	NEG	NEG	AR	bFGF	b-NGF	EGF	EGF R	FGF-4	FGF-6	FGF-7
B	Pos	Pos	NEG	NEG	AR	bFGF	b-NGF	EGF	EGF R	FGF-4	FGF-6	FGF-7
C	GCSF	GDNF	GM-CSF	HB-EGF	HGF	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-6	IGF-I	IGF-I SR
D	GCSF	GDNF	GM-CSF	HB-EGF	HGF	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-6	IGF-I	IGF-I SR
E	IGF-Ⅱ	M-CSF	M-CSF R	NT-3	NT-4	PDGF Rα	PDGF Rβ	PDGF-AA	PDGF-AB	PDGF-BB	PlGF	SCF
F	IGF-Ⅱ	M-CSF	M-CSF R	NT-3	NT-4	PDGF Rα	PDGF Rβ	PDGF-AA	PDGF-AB	PDGF-BB	PlGF	SCF
G	SCF R	TGF-α	TGF-β	TGF-β2	TGF-β3	VEGF	VEGF R2	VEGF R3	VEGF-D	BLANK	BLANK	POS
H	SCF R	TGF-α	TGF-β	TGF-β2	TGF-β3	VEGF	VEGF R2	VEGF R3	VEGF-D	BLANK	BLANK	POS

Control

	1	2	3	4	5	6	7	8	9	10	11	12
A	3241.35	3658.33	65.34	–	32.93	16.80	33.35	70.85	16.06	52.79	60.90	58.87
B	3391.73	3514.09	77.26	–	32.02	23.25	20.97	74.63	12.42	47.37	43.47	58.75
C	97.16	109.71	33.85	50.14	30.94	27.34	51.04	22.63	34.19	22.84	60.41	78.83
D	23.27	35.29	6.84	44.90	21.59	30.33	50.71	27.75	33.24	27.06	81.59	118.58
E	71.11	30.89	75.17	39.45	38.95	12.28	19.65	43.07	66.52	62.32	102.03	98.57
F	64.87	27.03	80.08	36.88	35.48	23.20	31.15	44.26	54.89	50.06	113.47	181.24
G	31.84	9.62	23.26	76.25	53.35	49.23	43.24	103.74	100.40	22.41	135.56	3464.66
H	25.55	6.57	23.98	75.62	35.60	28.50	31.43	107.61	99.60	22.71	128.46	3483.39

TPO

	1	2	3	4	5	6	7	8	9	10	11	12
A	2774.79	2845.69	46.33	–	20.86	15.94	18.77	76.69	15.64	42.14	27.28	58.47
B	2674.86	2670.53	49.58	–	22.77	16.77	13.83	77.10	13.46	33.63	31.10	54.49
C	110.96	103.87	15.42	39.44	19.10	22.06	37.96	23.41	31.37	18.72	54.44	71.03
D	29.03	47.72	7.44	36.54	21.10	21.09	44.82	27.08	41.54	27.03	60.60	86.58
E	74.16	27.93	60.45	31.83	28.90	16.23	25.71	35.14	52.58	40.37	63.82	48.63
F	51.46	21.11	51.82	29.47	30.74	17.67	16.28	36.01	44.60	32.99	76.80	96.06
G	18.06	4.73	18.11	59.26	36.19	27.09	26.31	94.06	89.27	0.01	45.05	3165.35
H	15.91	3.16	15.55	57.32	37.37	22.92	17.34	85.19	110.73	0.96	38.95	2785.42

ETP

	1	2	3	4	5	6	7	8	9	10	11	12
A	3103.24	3099.70	44.24	–	29.89	26.72	32.46	116.27	43.74	61.53	72.77	85.09
B	3066.02	3035.39	33.86	–	26.43	24.29	32.86	108.23	32.47	51.79	53.77	80.94
C	76.61	83.06	6.74	41.07	28.59	32.05	69.56	32.20	39.23	31.70	67.87	109.42
D	17.85	31.75	5.70	36.85	18.78	33.21	66.11	34.32	42.12	39.28	84.84	118.51
E	29.68	20.25	53.17	30.71	33.12	18.48	20.18	34.35	62.75	74.10	107.91	103.22
F	51.92	17.50	53.73	29.41	35.15	18.71	18.09	30.83	52.50	61.80	93.60	128.89
G	20.99	3.43	18.05	58.84	46.05	47.88	40.27	97.15	103.32	13.81	108.52	3233.61
H	13.39	5.09	16.02	59.81	48.21	33.10	57.85	108.46	96.68	15.50	113.40	3187.90

LTP

	1	2	3	4	5	6	7	8	9	10	11	12
A	2881.22	3037.58	98.60	–	26.90	34.17	34.24	95.72	15.45	15.29	14.59	34.94
B	2678.82	2883.56	72.73	–	22.92	30.53	27.64	85.79	9.09	16.38	17.21	38.04
C	112.38	128.14	21.84	35.74	17.83	32.03	68.50	25.15	28.73	9.55	36.81	57.58
D	17.67	39.22	7.93	32.86	15.66	18.26	62.26	29.57	36.10	27.12	61.38	75.58
E	81.20	39.28	89.11	41.35	29.76	9.22	20.75	39.36	58.35	51.02	82.69	76.19
F	71.79	22.18	64.18	32.79	36.68	22.05	29.93	39.22	56.56	47.15	99.46	149.22
G	27.37	7.31	25.35	64.42	49.49	43.04	43.15	110.32	103.57	23.78	115.72	2897.54
H	27.87	8.26	24.01	58.53	40.43	48.13	44.90	103.00	96.43	29.56	113.91	2802.79

4. Discussion

TPO-RA is an attractive treatment option for MDS patients with thrombocytopenia requiring frequent platelet transfusions. In a previous phase 1/2 study, ETP significantly improved platelet counts and reduced bleeding events in patients with low-risk MDS[31]. In addition, there was no significant difference in the rate of progression to AML between the ETP arm and placebo arm. In addition, a phase 2 study demonstrated that ETP was effective for reducing thrombocytopenic events without a new safety concern in advanced MDS and AML, while it didn't improve hematologic parameters or overall survival[32]. Similarly, Rom increased platelet counts, decreased bleeding events, and reduced platelet transfusions in patients with low and intermediate-risk MDS in phase 1/2 studies[33]. Whereas AML rates were similar between Rom arm and placebo arm (AML rate at 58 weeks: Rom 6% *vs*. placebo 4.9%; HR, 1.20; 95% CI, 0.38-3.84), Rom was discontinued due to the initial concern about AML risk[33]. In addition, a recent meta-analysis on 8 eligible studies including 1,047 MDS patients receiving TPO-RA (ETP or Rom) *vs*. placebo demonstrated TPO-RA was effective for reducing bleeding events without a significant increase of AML[34]. However, TPO-RA reduced the overall response rate (ORR) for unknown reasons, especially in the ETP-treated group and high-risk MDS group. So, further studies are needed to evaluate the efficacy and safety of TPO-RAs in MDS patients.

LTP is a novel TPO-RA, which is used for the treatment of thrombocytopenia associated with hepatic diseases planned to receive invasive procedures[35,36]. In this study, we conducted several preclinical experiments to examine the possibility of LTP as a treatment option for MDS patients with thrombocytopenia. As a result, we found that LTP at 3 μM and 10 μM promoted the growth of megakaryocytic cells from MDS $CD34^+$ cells as efficiently as 3 μM of ETP and rhTPO. However, the most effective reagents were different in individual cases. So, this analysis may be useful to select the most effective TPO-RA. However, it should be verified whether the result of this analysis is clinically applicable or not.

In contrast, although ETP at 10 μM did not affect colony-forming activities or self-renewal activities of $CD34^+$ cells in the replating assay, 10 μM of ETP inhibited megakaryocytic growth of both normal and MDS $CD34^+$ cells. As for this mechanism, we found that ETP at 10 μM promoted apoptosis of cultured $CD34^+$ cells compared with rhTPO. This result is consistent with the previous report that ≥10 μM ETP induced cell toxicities in the TPO-dependent BAF3 cell line engineered to express human c-Mpl and

in several AML and lymphoma cell lines[30,36]. In addition, ETP was shown to exert antileukemic effects through its ability to bind to iron and the modulation of intracellular iron homeostasis[37,38]. Because this concentration is easily achieved in patients receiving the approved dosage of ETP[29], special attention has to be paid when using ETP. However, these toxic effects were mediated independently of the binding of ETP to c-Mpl. Therefore, such toxic effects are considered to be specific for ETP but not associated with Rom or LTP. Because LTP did not show these inhibitory activities in our in *vitro* studies, LTP may be safer than ETP .

In this study, we also confirmed that LTP supports megakaryocytic differentiation as effectively as ETP and rhTPO do. In these experiments, a predominant growth of immature blast cells was not observed after the cells were cultured with LTP. This result was also confirmed by the decrease of $CD34^+$ cell fraction after the liquid culture with LTP, suggesting that LTP may be given to MDS patients without increasing the risk of progression to AML. However, it is important to consider the fact that MDS cells are composed of several clones with different genetic abnormalities and disease progression from MDS to AML often originates from a minor clone. Therefore, further studies are required to draw a definite conclusion as to the risk of AML development by LTP.

Another concern associated with the long-term treatment with these TPO-RAs is the potential induction of myelofibrosis, which may occur via the induction of various cytokines such as TGF-β1 and platelet-derived growth factor (PDGF) that are secreted during the process of excessive Mks[39-41]. In this study, TGF-β was not stimulated by any TPO-RAs, however, the secretion of FGF-7, also known as keratinocyte growth factor and a member of the FGF family, was stimulated by ETP but reduced by LTP. Importantly, FGF-7specifically acts on epithelial cells, thereby being involved in wound healing and lung fibrosis[42]. Although we were not able to find any previous report supporting the role of FGF-7 in myelofibrosis, it is worth to investigate the systemic effects of FGF-7 and its potential implication in the pathogenesis of myelofibrosis.

We here found that LTP revealed some different biologic activities from those of ETP in terms of megakaryocytic growth of $CD34^+$ cells and cytokine secretion from BM MNCs. These differences were supposed to result from c-Mpl-dependent and independent mechanisms. Further mechanistic and clinical studies are required to determine which TPO-RA is the most appropriate for MDS and for each patient.

5. Conclusions

Our preclinical data indicate that LTP would be as effective as ETP in promoting the growth and differentiation of Mks that developed from MDS $CD34^+$ cells. So, LTP would be a useful treatment option for MDS patients with thrombocytopenia.

Author Contributions:

H.T., and I.M. designed and supervised research.
K.S., A.F., R.F., and H.T. analyzed the data.
K.S., and H.T. provided disease-specific analysis.
K.S., J.L.E., and H.T. performed statistical analysis.
K.S., J.L.E., T.K., K.S., and H.T. prepared figures and tables.
K.S., H.T., J.L.E., and I.M. wrote the manuscript.
Y.M., H.H, and K.T. revised the manuscript.

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Conflicts of Interest:

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The remaining authors declare no competing financial interests.

References

1. Kaushansky K. (2015) Thrombopoiesis. Semin Hematol 52: 4-11

2. Noetzli LJ, et al. (2019) New Insights Into the Differentiation of Megakaryocytes From Hematopoietic Progenitors. Arterioscler Thromb Vasc Biol 39: 1288-1300

3. Li J, et al. (2001) Thrombocytopenia caused by the development of antibodies to thrombopoietin. Blood 98: 3241-3248

4. Basser R, et al. (2002) The impact of thrombopoietin on clinical practice. Curr Pharm Des 8: 369-77

5. Kuter DJ. (2007) New thrombopoietic growth factors. Blood 109: 4607-4616

6. Clemons Bankston P, et al. (2019) New Small Molecule Drugs for Thrombocytopenia: Chemical, Pharmacological, and Therapeutic Use Considerations. Int J Mol Sci 20: 3013

7. Bussel J, et al. (2019) Mechanisms and therapeutic prospects of thrombopoietin receptor agonists. Semin Hematol 56: 262-278

8. Kim ES. (2016) Lusutrombopag: First Global Approval. Drugs 76: 155-158

9. de Sauvage FJ, et al. (1996) Physiological regulation of early and late stages of megakaryocytopoiesis by thrombopoietin. J Exp Med 183: 651-656

10. Murone M, et al. (1998) Hematopoietic deficiencies in c-mpl and TPO knockout mice. Stem Cells 16: 1-6

11. Qian H, et al. (2007) Critical role of thrombopoietin in maintaining adult quiescent hematopoietic stem cells. Cell Stem Cell 1: 671-684

12. Yoshihara H, et al. (2007) Thrombopoietin/MPL signaling regulates hematopoietic stem cell quiescence and interaction with the osteoblastic niche. Cell Stem Cell 1: 685-697

13. Fox N, et al. (2002) Thrombopoietin expands hematopoietic stem cells after transplantation., J Clin Invest 110: 389-394

14. Townsley DM, et al. (2017) Eltrombopag Added to Standard Immunosuppression for Aplastic Anemia.
N Engl J Med 376: 1540-1550

15. Yoshizato T, et al. (2015) Somatic mutations and clonal hematopoiesis in aplastic anemia. N Engl J Med 373: 35-47

16. Genovese G, et al. (2014) Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. N Engl J Med 371: 2477-2487

17. Espinoza JL, et al. (2017) Microbe-Induced Inflammatory Signals Triggering Acquired Bone Marrow Failure Syndromes. Frontiers in immunology 8: 186

18. Gondek LP, et al. (2020) Assessing clonal haematopoiesis: clinical burdens and benefits of diagnosing myelodysplastic syndrome precursor states. Lancet Haematol 7: e73-e81

19. Shumilov E, et al. (2018) Current status and trends in the diagnostics of AML and MDS.
Blood Rev 32: 508-519

20. Kennedy JA, et al. (2017) Clinical Implications of Genetic Mutations in Myelodysplastic Syndrome. J Clin Oncol 35: 968-974

21. Lindsley RC, et al. (2017) Prognostic Mutations in Myelodysplastic Syndrome after Stem-Cell Transplantation. N Engl J Med 376: 536-547

22. Lindsley RC, et al. (2017) Prognostic Mutations in Myelodysplastic Syndrome after Stem-Cell Transplantation. N Engl J Med 376: 536-547

23. Chandhok NS, et al. (2020) Hypomethylating agent based combinations in higher risk myelodysplastic syndrome. Leuk Lymphoma 61: 1012-1027

24. Sanz GF. (2019) In MDS, is higher risk higher reward? Hematology Am Soc Hematol Educ Program 381-390

25. Prica A, et al. (2014) Safety and efficacy of thrombopoietin-receptor agonists in myelodysplastic syndromes: a systematic review and meta-analysis of randomized controlled trials. Br J Haematol 167: 626-638

26. Hasserjian RP, et al. Myelodysplastic syndromes. Swerdlow SH, et al. (2017) WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues, Lyon, IARC, pp98-106.

27. Will B, et al. (2009) Effect of the nonpeptide thrombopoietin receptor agonist Eltrombopag on bone marrow cells from patients with acute myeloid leukemia and myelodysplastic syndrome Blood 114: 3899-3908.

28. Kanda Y. (2013) Investigation of the freely available easy-to-use software 'EZR' for medical statistics Bone Marrow Transplantation 48: 452-458

29. Deng Y, et al. (2011) Metabolism and disposition of eltrombopag, an oral, nonpeptide thrombopoietin receptor agonist, in healthy human subjects. Drug Metab Dispos 39: 1734-1746

30. Erickson-Miller CL, et al. (2009) Preclinical activity of eltrombopag (SB-497115), an oral, nonpeptide thrombopoietin receptor agonist. Stem Cells 227: 424-430

31. Oliva EN, et al. (2017) Eltrombopag versus placebo for low-risk myelodysplastic syndromes with thrombocytopenia (EQoL-MDS): phase 1 results of a single-blind, randomised, controlled, phase 2 superiority trial. Lancet Haematol 4:e127–136

32. Mittelman M, et al. (2018) Eltrombopag for advanced myelodysplastic syndromes or acute myeloid leukaemia and severe thrombocytopenia (ASPIRE): a randomised, placebo-controlled, phase 2 trial. Lancet Haematol 5: e34-e43

33. Giagounidis A, et al. (2014) Results of a randomized, double-blind study of romiplostim versus placebo in patients with low/intermediate-1-risk myelodysplastic syndrome and thrombocytopenia. Cancer 120: 1838-1846

34. Meng F, et al. (2020) Safety and Efficacy of Eltrombopag and Romiplostim in Myelodysplastic Syndromes: A Systematic Review and Meta-Analysis. Front Oncol. 10: 582686

35. Gilreath J, et al. (2021) Thrombopoietin Receptor Agonists (TPO-RAs): Drug Class Considerations for Pharmacists. Drugs 81: 1285-1305

36. Erickson-Miller CL, et al. (2010) Reduced proliferation of non-megakaryocytic acute myelogenous leukemia and other leukemia and lymphoma cell lines in response to eltrombopag. Leuk Res 34: 1224-1231

37. Vlachodimitropoulou E, et al. (2017) Eltrombopag: a powerful chelator of cellular or extracellular iron(III) alone or combined with a second chelator. Blood 130: 1923-1933

38. Pallaud C, et al. (2018) Thrombopoietin receptor-independent stimulation of hematopoietic stem cells by eltrombopag. Transl Med 10: eaas9563

39. Zhao M, et al. (2014) Megakaryocytes maintain homeostatic quiescence and promote post-injury regeneration of hematopoietic stem cells. Nat Med 20: 1321-1326

40. Gangat N, et al. (2020) Myelofibrosis biology and contemporary management. Br J Haematol 191: 152-170

41. Zahr AA, et al. (2016) Bone marrow fibrosis in myelofibrosis: pathogenesis, prognosis and targeted strategies. Haematologica 101 :660-671

42. Yao L, et al. (2013) Protection against hyperoxia-induced lung fibrosis by KGF-induced MSCs mobilization in neonatal rats. Pediatr Transplant 17: 676-682

Figure legends

Fig. 1. Effects of LTP, ETP, and rhTPO on the growth of CD34⁺ cells from MDS cases

A, B. $CD34^+$ BM cells isolated from one healthy donor (A) and 7 MDS cases (B) were suspended at 1.0×10^4 cells/ml in triplicate and cultured for 7 or 14 days in StemSpan™ SFEM medium supplemented with rhIL-3, rhIL-6, and rhSCF and rhFlt3L. Viable cell numbers were calculated at the indicated points with the Cell Titer-Glo® Luminescent Cell Viability assay. Data are presented as mean fold expansion.

Fig. 2. CFU-Mk assays to evaluate the effects of ETP, LTP, and rhTPO on the growth and megakaryocytic differentiation of CD34⁺ cells from MDS cases

A. $CD34^+$ BM cells from MDS cases (n=5) were cultured in MegaCult™-C kit medium supplemented with IL-3 and IL-6 at a density of 1.0×10^5 cells/ml in the presence of rhTPO, ETP, or LTP at the indicated concentrations , or in the absence of TPO-RAs (control culture, CTL) for 14 days. Colonies were observed under microscopy. Megakaryocyte (Mk) colonies were identified by an anti-human GPⅡb/Ⅲ (CD41a) antibody (Ab). Morphology of colony-forming cells was analyzed by the May-Grunwald-Giemsa staining on cytospin preparations.
B. Total number of Mk colonies were counted. Data are presented as mean ± SD (n=5).

C. Numbers of small, medium, large, and mixed colonies were counted. Data are presented as mean ± SD (n=5).

Fig. 3. Effects of ETP, LTP, and rhTPO on megakaryocytic differentiation and cell survival of MDS CD34+ cells

A, B. CD34+ from MDS patients (n=6) were cultured in StemSpan™ SFEM medium supplemented with rhIL-3, rhIL-6, rhSCF and rhFlt3L in the presence of 100 ng/ml rhTPO, 10 μM or 3μM of ETP with 5 μM CuSO4, or 3 μM or 10 μM LTP. After 7-day cultures, the percentages of $CD45^+CD34^+$ (A) and $CD41^+$ (B) cells were analyzed by flow cytometry. Data

are presented as mean ± SD.

C. In the same condition, the percentages of Annexin V positive and 7-AAD negative apoptotic cells were evaluated. Data are presented as mean ± SD.

Fig. 4. Effects of LTP, ETP and rhTPO on long-term self-renewal activities of $CD34^+$ cells from MDS cells.

A. An outline of the CFU replating assays is shown. MDS $CD34^+$ BM cells were suspended into methylcellulose medium with or without rhTPO, ETP, or LTP at a density 1,000 cells/ml in duplicate. The numbers of CFUs were counted on day 12-14. Then, colony-forming cells were harvested and replated into methylcellulose medium for the next CFU assays under the same conditions. These procedures were repeated until no colonies formed.

B. CFU replating assays were performed with MDS $CD34^+$ BM cells (n=18) with or without $CuSO_4$, rhTPO, ETP, or LTP at indicated concentrations. Data are presented as mean ± SD.