Stimulation of GPR15 on vascular endothelial cells mediates angiogenesis and cytoprotective function

Masahiko Fukatsu[1*], Xintaow Wang[1*], Lobna Alkebsi[1], Hiroshi Ohkawara[1], Goichi Honda[2], Takayuki Ikezoe[1]

1 Department of Hematology, Fukushima Medical University, Fukushima, Japan
2 Department of Medical Affairs, Asahi Kasei Pharma, Tokyo, Japan.
* Both authors contributed equally to this work.

ISTH MELBOURNE 2019 JULY6-10 RESEARCH DISCOVERY OUTCOMES
ISTH2019.ORG

INTRODUCTION

- **GPR15** is an orphan G protein-coupled receptor expressed on lymphocytes as well as vascular endothelial cells (VECs).
- Recently, other researchers have identified **natural ligand for GPR15 (GPR15L),** which was coded by human gene *C10ORF99* or murine *2610528A11Rik* (ref. 1).
- We previously found that **thrombomodulin**, a natural anticoagulant expressed on cell surface of VECs, induced angiogenesis and protected tacrolimus-induced VEC apoptosis via GPR15-dependent manner (ref. 2-3).
- The present study explored whether GPR15L causes angiogenic and cytoprotective function similar to thrombomodulin over VECs.

METHOD

- Human as well as murine GPR15L was synthesized by Biologica Co. (Nagoya, Japan).
- Human umbilical vein endothelial cells (HUVECs) were cultured with either human GPR15L, vascular endothelial growth factor (VEGF) or control diluent. Their proliferation was measured by BrdU incorporation assay after 24h culture.
- The tube formation assays were also utilized to asses the angiogenic potential of GPR15L.
- Western blot analysis was employed to evaluate the effects of GPR15L on pro-survival signal transduction pathways.
- To assess the effects of GPR15L *in vivo*, growth factor-reduced Matrigel, with control diluent, murine GPR15L or VEGF was subcutaneously inject into C57BL/6 wild type (WT) or GPR15 knockout (KO) mice near the abdominal midline. Six days later, mice were euthanized, and the matrigel plugs were dissected out and photographed.

RESULTS

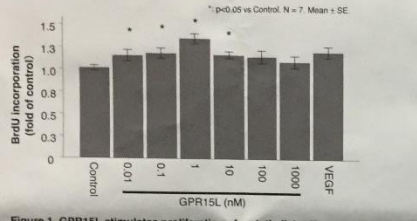
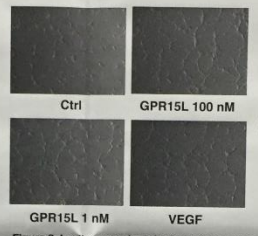


Figure 1. GPR15L stimulates proliferation of endothelial cells.
BrdU incorporation assay. HUVECs were cultured either with GPR15L or VEGF for 24h. Proliferation was measured by BrdU incorporation assays.



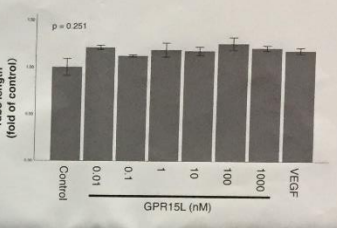


Figure 2. In vitro vascular tube formation assays.
HUVECs were plated on growth factor-reduced matrigel-precoated and incubated with control diluent, GPR15L (0.01-1000 nM) or VEGF (0.5 nM). After 6 h, the endothelial cell-derived tube-like structure was photographed (3 random fields for each condition, representative images in left panel) and analyzed with ImageJ software (right panel).

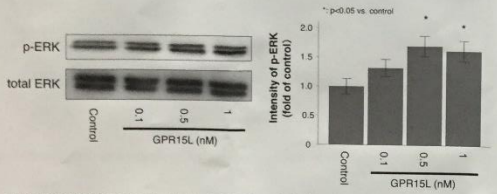


Figure 3. GPR15L increases the levels of p-ERK in endothelial cells.
(Left panel) HUVECs were exposed to control diluents (PBS) or GPR15L (0.1, 0.5 or 1 nM). After 15 min, proteins were extracted and subjected to western blot analyses. (Right panel) Relative quantifications of p-ERK. ImageJ software was used to measure the band intensities after western blotting. All experiments were performed 3 times.

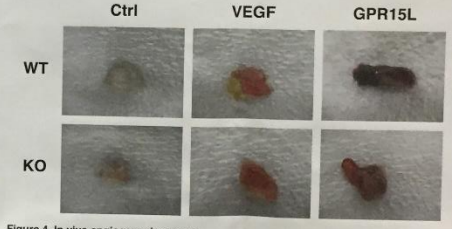


Figure 4. In vivo angiogenesis assays.
Growth factor-reduced Matrigel (0.5 mL), containing heparin (40 U/mL), either with control diluent, GPR15L or VEGF (20 ng/mL) was subcutaneously injected into WT or GPR15 KO mice near the abdominal midline. Six days after injection, mice were euthanized, and the Matrigel plugs were surgically removed. Representative images are shown.

CONCLUSIONS

- GPR15 and its ligand contribute to angiogenesis possibly through stimulatation of pro-survival signal of VECs as demonstrated in BrdU incorporation assay and western blot analysis.
- Although not statistically significant, similar trends were observed in *in vitro* vascular tube formation and *in vivo* angiogenesis assays.
- As in our previous studies, ligands for GPR15 may be a promising agent to prevent potentially lethal hematopoietic stem cell transplantation (HSCT)-related complications such as thrombotic microangiopathy and sinusoidal obstruction syndrome, where insults of VECs play a pathogenic role.
- Future directions include characterizing cytoprotective function of GPR15L under stress conditions, signaling pathway downstream of GPR15, and mouse model approach to prove its efficacy in HSCT.

REFERENCES

1. Suply T, Hannedouche S, Carte N, et al. A natural ligand for the orphan receptor GPR15 modulates lymphocyte recruitment to epithelia. *Sci Signal.* 2017;10:1.
2. Pan B, Wang X, Nishioka C, et al. G-protein coupled receptor 15 mediates angiogenesis and cytoprotective function of thrombomodulin. *Sci Rep.* 2017;7:692.
3. Wang X, Pan B, Honda G, et al. Cytoprotective and pro-angiogenic functions of thrombomodulin are preserved in the C loop of the fifth epidermal growth factor-like domain. *Haematologica.* 2018;103:1730.

ACKNOWLEDGEMENTS

This study was supported by Asahi Kasei Pharma (Tokyo, Japan), JSPS KAKENHI (Grant Number JP18H02844), and Uehara Memorial Foundation.

CONTACT INFORMATION

Correspondence: Takayuki Ikezoe

Department of Hematology, Fukushima Medical University, 1 Hikarigaoka, Fukushima 960-1295, Japan.

E-mail address: ikezoet@fmu.ac.jp.

ISTH MELBOURNE 2019 JULY6-10 RESEARCH DISCOVERY OUTCOMES
ISTH2019.ORG

Circulating intranuclear proteins may play a role in development of coagulopathy following remission induction chemotherapy in individuals with acute leukemia

K. HARADA[1], X. WANG[1], M. FUKATSU[1], H. TAKAHASHI[1], A. SHICHISHIMA[1], S. KIMURA[1], H. OHKAWARA[1], S. YAMADA[2], T. ITO[3], T. IKEZOE[1]

[1]Department of Hematology, Fukushima Medical University, Fukushima, Japan.
[2]R&D Center, Shino-Test Corporation, Sagamihara, Japan.
[3]Department of Systems Biology in Thromboregulation, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan.

CONTACT INFORMATION ks1022@fmu.ac.jp

INTRODUCTION

- Previous studies found that approximately 15% of acute myeloid leukemia (AML) patients developed disseminated intravascular coagulation (DIC) soon after the initiation of remission induction chemotherapy[1].
- This suggested the crosslink between coagulopathy and tumor lysis, although detailed mechanisms by which tumor lysis causes hyper coagulation remain unknown.
- Recently, intranuclear proteins including high mobility group box 1 (HMGB1) and histone H3 released from activated neutrophils were shown to be involved in the pathogenesis of DIC in patients with sepsis[2].
- HMGB1 stimulated thrombin-induced thrombus formation in rats[3]. Histones, one of the main components of neutrophils extracellular traps (NETs) activate coagulation pathways and platelets[4, 5]. In vitro studies found that histone H3 and H4 induced apoptosis in vascular endothelial cells[6], which also links to the pathogenesis of DIC.

AIMS

- These observations prompted us to hypothesize that HMGB1 and histone H3 liberated from leukemia cells undergoing apoptosis after chemotherapy might play a role in development of DIC.
- We would like to test if this hypothesis is correct.

METHODS

- We prospectively measured plasma levels of coagulation markers and intranuclear proteins, including HMGB1 and histone H3, in patients with acute leukemia (n=17) who received chemotherapy (Table 1).
- DIC was diagnosed according to the diagnostic criteria published by Japanese Society on Thrombosis and Hemostasis in 2017 [7].

RESULTS

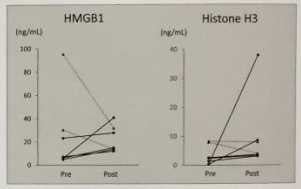
- DIC was diagnosed in ten out of 17 patients (58.8%) at the time of diagnosis of leukemia.
- We found that either six or one patients developed DIC or experienced deterioration of the coagulopathy, respectively, after administration of anti-leukemic agents (Table 2).
- Of note, an increase in plasma levels of HMGB1 and histone H3 after chemotherapy was noted in five patients out of these 7 patients (Figure 1).
- Curiously, elevation of intranuclear proteins occurred few days earlier than that of global coagulation markers such as D-dimer (Figure 2).
- Moreover, the use of recombinant human soluble thrombomodulin (rTM) promptly decreased levels of nuclear proteins in parallel with improvement of coagulopathy (Figure 2 and 3).

Table 1. Characteristics

Age: median 62 years old (29-74)	
Sex: Male 10, Female 7	
Subgroups: Acute leukemia	
AML	6
APL	4
ph- ALL	4
ph+ ALL	3
	17

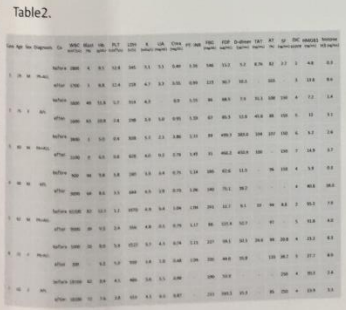
AML: acute myeloid leukemia, APL: acute promyelocytic leukemia, Ph-ALL: Philadelphia chromosome-negative acute lymphoblastic leukemia, Ph+ALL: Philadelphia chromosome-positive acute lymphoblastic leukemia

Figure 1.



Values of serum levels of HMGB1 and histone H3 in patients with acute leukemia who developed DIC or experienced deterioration of the coagulopathy after remission induction chemotherapy. Pre, before chemotherapy, Post, after chemotherapy.

Table2.



Ph-ALL: Philadelphia chromosome-negative acute lymphoblastic leukemia, Ph+ALL: Philadelphia chromosome-positive acute lymphoblastic leukemia, APL: acute promyelocytic leukemia, CY: cyclophosphamide hydrate, DIC: disseminated intravascular coagulation, DNR: daunorubicin hydrochloride, PLT: platelet, LDH: lactate dehydrogenase, UA: uric acid, Cre: creatinine, PT: prothrombin time, APTT: activated partial thromboplastin time, FBG: fibrinogen, FDP: fibrin/fibrinogen degradation products, D-dimer: D-dimer, TAT: thrombin-antithrombin complex, AT: antithrombin activity, SF: soluble fibrin, -: Not implemented

Figure 2.

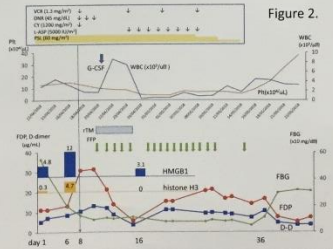


Figure2.
Case 1 (29 y) Ph (-) ALL. After the administration of prednisolone, elevations of intranuclear proteins occurred 2 days earlier than that of global coagulation markers such as D-dimer. After the administration of rTM, levels of nuclear proteins were decreased in parallel with improvement of coagulopathy.

VCR: vincristine sulfate, DNR: daunorubicin hydrochloride, CY: cyclophosphamide hydrate, L-ASP: L-asparaginase, PSL: prednisolone, G-CSF: granulocyte-colony stimulating factor, FDP: fibrin/fibrinogen degradation, D-D: D-dimer, FBG: fibrinogen, FFP: fresh frozen plasma, rTM: recombinant human soluble thrombomodulin

Figure 3.

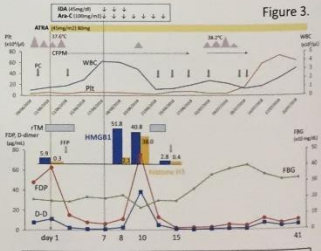


Figure3
Case4 (48 y) APL. Administration of All-trans retinoic acid (ATRA) resulted in an increase in WBC. The patient subsequently received induction chemotherapy with combination of IDA and AraC which caused an increase in levels of HMGB1 and histone H3 followed by exacerbation of coagulopathy. The use of rTM promptly decreased levels of nuclear proteins in parallel with improvement of coagulopathy.

IDA: idarubicin hydrochloride, AraC: cytarabine, ATRA: tretinoin, CFPM: Cefoperone Sulbactam Hydrate, FDP: fibrin/fibrinogen degradation product, D-D: D-dimer, FBG: fibrinogen, FFP: fresh frozen plasma, PC: Platelet Concentrate, rTM: recombinant human soluble thrombomodulin

CONCLUSIONS

These results suggested that remission induction chemotherapy caused apoptosis of leukemia cells, leading to release of intranuclear proteins which may caused DIC in association with vascular endothelial damage and direct activation of platelets as well as coagulation pathways.

REFERENCES

1. **Uchiumi H et al.** Prevalence and clinical characteristics of acute myeloid leukemia associated with disseminated intravascular coagulation. *Int J Hematol. 2007; 86: 137-42*
2. **Okamoto K et al.** Sepsis and disseminated intravascular coagulation. *J Intensive Care. 2016; 4: 23. doi: 10.1186/s40560-016-0149-0*
3. **Ito T et al.** High-mobility group box 1 protein promotes development of microvascular thrombosis in rats. *J Thromb Haemost. 2007;5:109-16*
4. **Carestia A et al.** Functional responses and molecular mechanisms involved in histone-mediated platelet activation. *Thromb Haemost. 2013; 110: 1035-45*
5. **Nakahara M et al.** Recombinant thrombomodulin protects mice against histone-induced lethal thromboembolism. *PLoS One. 2013;8:e75961*
6. **Xu J et al.** Extracellular histones are major mediators of death in sepsis. *Nat Med. 2009; 15: 1318-21*
7. **Wada H et al.** The approval of revised diagnostic criteria for DIC from the Japanese Society on Thrombosis and Hemostasis. *Thromb J. 2017;15:17. doi: 10.1186/s12959-017-0142-4*