D．考察

漢方は日本の臨床現場で、患者へ投与されている。血液学の分野では人参養栄湯、四物湯、十全大補湯、大防風湯は貧血患者へ処方されている。しかしながら、これら漢方薬の効果をもたらす分子メカニズム解明は発展途上である。この問題に取り組むため、漢方薬をin vitro培養へ添加し、IL-3, EPO, SCF等の造血サイトカインなしで培養した。４種類の漢方薬のうち、人参養栄湯は培養8日目でCD71(増殖マーカー)陽性生細胞数を増加させた。一方、十全大補湯は培養11日目でTer119(成熟赤血球マーカー)陽性細胞を増加させた。リアルタイムPCRによる赤血球造血関連遺伝子の解析で、人参養栄湯、十全大補湯添加培養後、変化が認められた。しかしながら、人参養栄湯、十全大補湯に比べ、四物湯、大防風湯添加で、細胞の表現型・遺伝子発現がわずかに変化した。今後、その分子メカニズム解明が期待される。

Ｅ．結論

マウス骨髄細胞へ人参養栄湯、四物湯、十全大補湯、大防風湯、４種類の漢方薬を添加培養し、造血へ与える影響を検討した。サイトカイン非存在下において、人参養栄湯は細胞増殖を促進し、十全大補湯は赤血球分化を促進する事が明らかとなった。

Ｆ．健康危険情報

国民の生命、健康に重大な影響を及ぼすと考えられる研究成果は得られていない。

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H．知的財産権の出願・登録状況
（予定を含む。）

1. 特許取得

特になし

2. 実用新案登録

特になし

3. その他

特になし

別紙4

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Sugiyama D, Kulkeaw K, Mizuochi C, Horio Y, Okayama S.	Hepatoblasts comprise a niche for fetal liver erythropoiesis through cytokine production	Biochem Biophys Res Commun			In press
Inoue T, Kulkeaw K, Okayama S, Tani K and Sugiyama D.	Variation in Mesodermal and Hematopoietic Potential of Adult Skin-derived Induced Pluripotent Stem Cell Lines in Mice.	Stem Cell Reviews and Reports			In press
Kulkeaw K, Ishitani T, Kanemaru T, Ivanovski O, Nakagawa M, Mizuochi C, Horio Y, Sugiyama D.	Cold exposure down-regulates zebrafish pigmentation.	Genes to Cells	16(4)	358-367	2011
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Sasaki T, Mizuochi C, Horio Y, Nakao K, Akashi K and Sugiyama D.	Regulation of hematopoietic cell clusters in the placental niche through SCF/c-Kit signaling in embryonic mouse.	Development	137(23)	3941-3952	2010

Title: Hepatoblasts comprise a niche for fetal liver erythropoiesis through cytokine production

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Abstract

In mammals, definitive erythropoiesis first occurs in fetal liver (FL), although little is known about how the process is regulated. FL consists of hepatoblasts, sinusoid endothelial cells and hematopoietic cells. To determine niche cells for fetal liver erythropoiesis, we isolated each FL component by flow cytometry. mRNA analysis suggested that Dlk-1-expressing hepatoblasts primarily expressed *EPO* and *SCF*, genes encoding erythropoietic cytokines. EPO protein was detected predominantly in hepatoblasts, as assessed by ELISA and immunohistochemistry, and was not detected in sinusoid endothelial cells and hematopoietic cells. To characterize hepatoblast function in FL, we analyzed *Map2k4*$^{-/-}$ mouse embryos, which lack hepatoblasts, and observed down-regulation of *EPO* and *SCF* expression in FL relative to wild-type mice. Our observations demonstrate that hepatoblasts comprise a niche for erythropoiesis through cytokine secretion.

Keywords: Hepatoblasts, Erythropoiesis, Fetal Liver

Introduction

Hematopoiesis is the process by which pluripotent hematopoietic stem cells (HSCs) are generated, differentiate into specific progenitors, and ultimately mature into numerous blood cell types, including erythrocytes, megakaryocytes, lymphocytes, neutrophils, and macrophages [1]. In the mouse embryo, HSCs and hematopoietic progenitors (HPCs) are generated in the aortic region, known as the para-aortic Splanchnopleura (p-Sp)/Aorta-Gonad-Mesonephros (AGM) region, the yolk sac (YS), and the placenta [2-9].In mid-gestation, hematopoiesis, particularly erythropoiesis, occurs in fetal liver (FL) [2,9,10]. Erythropoiesis has been classically described as occurring in two waves: first primitive and then definitive erythropoiesis [4]. Primitive erythropoiesis supports a transient wave of embryonic erythropoiesis in the yolk sac, while definitive erythropoiesis contributes to adult-type erythropoiesis. In mammals, definitive erythropoiesis occurs first in FL and then shifts to adult bone marrow (BM) shortly before birth [11]. There are greater numbers of erythroid progenitors, such as burst-forming unit-erythroids (BFU-E) and colony-forming unit-erythroids (CFU-E), in FL than in BM [12]. In addition, in mice, the number of mature erythroid cells in circulation dramatically increases from 12.5 to 16.5 day post-coitum (dpc), suggesting that massive expansion of both erythroid progenitors and terminally differentiated erythroid cells occurs in FL [13]. Erythropoietin (EPO) is a cytokine that regulates erythroid cell differentiation, maturation, proliferation and survival, and is primarily produced by adult kidney cells, where production is up-regulated by hypoxia [14].

Terminal proliferation and differentiation of CFU-E is stimulated by EPO, whereas BFU-E, which are more immature than CFU-E, respond to stem cell factor (SCF), insulin-like growth factor (IGF)-1, corticosteroids, interleukin (IL)-3 and IL-6, in addition to EPO [15]. Although FL is the most active organ for erythropoiesis, little is known about how erythropoiesis is regulated in that tissue.

Here, in order to identify niche cells for erythropoiesis, we used flow cytometry based on surface molecule expression to separate cells in early FL into hepatoblasts (HBs), sinusoid endothelial cells (SECs) and hematopoietic cells (HCs), and then evaluated cytokine expression in each fraction.

Materials and methods

Animals. ICR and C57BL/6J mice were purchased from Nihon SLC (Hamamatsu, Japan) and Kyudo (Tosu, Japan), respectively. *Map2k4*$^{+/-}$ mice were provided by RIKEN BioResource Center (Tsukuba, Japan). Noon of the day of the plug was defined as 0.5 day post-coitum (dpc). Embryos at 12.5 and 14.5 dpc were dissected in PBS under a stereomicroscope. Animals were handled according to Guidelines for Laboratory Animals of Kyushu University.

Flow cytometry. For hepatoblasts and sinusoid endothelial cells, fetal livers at 12.5 and 14.5 dpc were digested in 1mg/mL collagenase (Washington Biochem Co., Freehold, New Jersey) in alpha-MEM containing 20% FBS, filtered through 40-μm nylon mesh, and washed once with PBS. Cells were stained with a FITC-conjugated anti-mouse Dlk-1 Ab (MBL, Nagoya, Japan), a PE-conjugated anti-mouse Lyve-1 Ab (MBL), an APC-conjugated anti-mouse CD31 Ab (Biolegend, San Diego, CA), a PE-Cy7-conjugated anti-mouse CD45 Ab (eBioscience, San Diego, CA), and a PE-Cy7-conjugated anti-mouse Ter119 Ab (eBioscience).

Real time-PCR. RNA was extracted from sorted and fetal liver samples using a RiboPure™ kit (Life Technologies, Carlsbad, CA) and mRNA was reverse transcribed using a High-Capacity RNA-to-cDNA kit (Life Technologies). cDNA synthesis quality was evaluated by amplifying mouse *β-actin* by PCR. Thirty thermal cycles were employed as follows: denaturation at 95°C for 10 seconds, annealing at 60°C for 20

seconds, and extension at 72°C for 20 seconds. Gene expression levels were measured by real time-PCR with TaqMan® Gene Expression Master Mix and StepOnePlus™ real time PCR (Life Technologies). All probes (*Flt3-L, TPO, EPO, SCF, IL-3, IL-6, IL-11, G-CSF* and *GM-CSF*) were from TaqMan® Gene Expression Assays (Life Technologies). All samples were assayed in triplicate wells. mRNA levels were normalized to *β-actin* and the relative quantity (RQ) of expression was compared with a reference sample.

Enzyme-Linked Immunosorbent Assay (ELISA). Lysates of sorted cells were obtained using a cell lysis buffer (M-Per® Mammalian Protein Extraction Reagent, Thermo Fisher Scientific, Waltham, MA) containing protease inhibitors (Protease Inhibitor Cocktail, Sigma Aldrich, St. Louis, MO). The sample was centrifuged at 14000xg at 4°C for 15 minutes. Supernatants containing soluble protein were collected and protein concentration was estimated by measuring absorbance at 280 nm (NANODROP 2000C, Thermo Fisher Scientific). SCF and EPO in sorted cells were assayed using an ELISA kit (Mouse SCF Immunoassay and Mouse EPO Immunoassay, R&D systems) according to the manufacturer's instructions. Experiments were performed in duplicate. The O.D. was measured using a Thermo Multiskan EX plate reader (Thermo Fisher Scientific).

Immunohistochemistry. Dissected ICR mouse embryos were fixed in 2% paraformaldehyde in PBS overnight at 4°C and washed in PBS three times. After 27% sucrose infusion, embryos were embedded in OCT compound (Sakura Finetek, Tokyo, Japan) and frozen in liquid nitrogen (vapor phase). Frozen embryos were sectioned at 20μm, transferred onto glass slides (Matsunami, Osaka, Japan), and dried. After blocking in 1% BSA in PBS, sections were incubated with primary antibodies overnight at 4°C. Anti-mouse Dlk-1 Ab (MBL), anti-mouse Lyve-1 Ab (MBL), anti-mouse c-Kit Ab (R&D Systems), anti-mouse SCF Ab (Santa Cruz Biotechnology, Santa Cruz, CA), anti-mouse EPO Ab (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-mouse Ki-67 Ab (Dako Corporation, Carpinteria, CA) served as primary antibodies. After washing in PBS three times, sections stained with Dlk-1, Lyve-1, c-Kit or Ki-67 antibodies were incubated with secondary antibodies and TOTO-3 (Life Technologies) for nuclear staining. In samples stained with SCF or EPO antibodies, the TSA Biotin System (PerkinElmer, Covina, CA) was used to amplify the signal. Donkey anti–rabbit IgG-Alexa555, Donkey anti–goat IgG-Alexa488, Donkey anti–rat IgG-Alexa488 and Alexa546 and Streptavidin Alexa546 (all from Life Technologies) served as secondary antibodies. Coverslips were mounted using fluorescent mounting medium (Dako Corporation, Carpinteria, CA). Slides were observed using a FV-1000 confocal microscope (Olympus, Tokyo, Japan).

Single cell preparation and cell counting. To prepare single cell suspensions of HCs from *Map2k4*$^{-/-}$ and wild-type FL at 12.5 dpc, dissected FL was crushed on the 40μm nylon cell strainer (BD Falcon, Bedford, MA) with the inner of 2.5 mL syringe. Cells were washed in PBS and collected into the tube. The number of living cells was counted after Trypan Blue staining.

Results

Cytokine expression in hepatoblasts

We examined expression of several cytokine genes in FL tissue samples at 12.5 and 14.5 dpc using real-time PCR. Significant expression of *FMS-like tyrosine kinase 3 ligand* (*Flt3l*), *thrombopoietin* (*TPO*), *erythropoietin* (*EPO*) and *interleukin-6* (*IL-6*) was seen in FL (Fig. 1A). *Dlk* (Delta-like 1 homolog) is a marker of hepatoblasts (HBs) in FL [16], while Lyve-1 (lymphatic vessel endothelial hyaluronan receptor 1) marks sinusoid endothelial cells (SECs) [17]. As shown in Figure 1B, fractions of HBs, SECs and hematopoietic cells (HCs) were isolated from mouse FL at both 12.5 and 14.5 dpc by flow cytometry based on expression of the following surface markers: (1) HBs, CD45–/Ter119–/Dlk-1+; (2) SECs, CD45–/Ter119–/Lyve-1+/CD31+; and (3) HCs, CD45+/Ter119+. To determine which component contributes to cytokine production, we used real-time PCR to examine *EPO, Flt3l, IL-6, SCF* and *TPO* expression in isolated HBs, SECs and HCs (Fig. 1C). *EPO* and *TPO* were expressed predominantly in HBs both at 12.5 dpc and 14.5 dpc, suggesting that erythropoiesis and megakaryopoiesis are activated by HBs in FL. Levels of *SCF* mRNA were higher in HBs than in SECs. Expression of *Flt3l* and *IL-6* was predominantly detected in HCs, suggesting that HSCs and HPCs expand via an autocrine mechanism. To investigate expression of the cytokine proteins EPO and SCF, we undertook Enzyme-Linked ImmunoSorbent Assay (ELISA) and found that EPO protein was predominantly detected in HBs (63.8 pg/mL/10000 cells) but was not detected in SECs and HCs (Fig. 2A). SCF protein, however, was detected in all fractions (Fig. 2B). In agreement with

SCF mRNA expression, HBs expressed SCF protein at a higher level (7.01 pg/mL/10000 cells) than did SECs (2.38 pg/mL/10000 cells) or HCs (0.025 pg/mL/10000 cells). When we compared expression levels of EPO and SCF proteins in each fraction, EPO expression was highest in HBs, suggesting that HBs secrete EPO to regulate FL erythropoiesis (Fig. 2C). To confirm EPO and SCF protein localization in FL, we performed immunohistochemistry and found that EPO protein was expressed primarily in Dlk-1-expressing HBs (Fig. 2D), in agreement with ELISA analysis. Staining of FL with anti-SCF antibody revealed SCF protein in both SECs expressing Lyve-1 and HBs expressing Dlk-1 (Fig. 2E-F).

Fetal liver of Map2k4$^{-/-}$ mouse embryos

To characterize HB function in FL, we analyzed *Map2k4* (*mitogen-activated protein kinase kinase 4*, formerly known as *Sek1* and *MKK4*)$^{-/-}$ mouse embryos, which lack FL HBs [18-20]. Real-time PCR analysis of whole FL from mutant and wild-type embryos showed that among cytokine-encoding genes, *EPO* and *SCF* mRNAs were down-regulated in the mutant mice, implying an impairment in hematopoiesis, particularly erythropoiesis, in *Map2k4* mutant mice (Fig. 3A). To evaluate potential alterations in hematopoiesis, we performed immunohistochemistry and found that the number of c-Kit expressing cells which represent HSCs and HPCs in the FL at this stage, decreased in a FL field from *Map2k4*$^{-/-}$ mouse embryo relative to wild-type embryo (Fig. 3B; green) [23]. In addition, the number of cells expressing Ki-67, a marker of cell proliferation, decreased in a FL field from *Map2k4*$^{-/-}$ mouse embryos

compared to wild-type embryos (Fig. 3B; red). In agreement with the Ki-67 staining, there were fewer FL cells seen in *Map2k4*$^{-/-}$ versus wild-type embryos (Fig. 3C).

Discussion

In FL, HSCs differentiate into mature HCs, particularly erythroid cells [9,10]. SCF and EPO are representative cytokines that regulate erythropoiesis [14,15]. Expression levels of *SCF* and *EPO* genes were higher in whole FL tissue than in adult BM and kidney, suggesting that FL primarily functions in erythropoiesis [24]. Recently, using a competitive repopulating assay, Chou and Lodish reported that FL stromal cells expressing both SCF and Dlk-1 support HSC maintenance [25]. However, it remained unclear which cells secreted EPO in FL. HBs are regarded as common progenitors of hepatocytes and biliary epithelial cells and thought to support liver construction through formation of a mesh-like structure [16,26]. Our results are strongly indicative of an additional role of HBs in producing erythropoietic cytokines SCF and EPO. Although gene expression of *IL-6* was observed in FL at 12.5 dpc, that was not detected in cell fractions of HBs, SECs and HCs (Fig. 1). As shown in Fig. 1B, we could observe unclassified cell fraction (CD45–/Ter119–/Dlk-1–/Lyve-1–/CD31–), based on surface marker expression, implying that this unclassified cell fraction may express *IL-6* gene. It will be further necessary to clarify roles of the unclassified cells in FL hematopoiesis in the future.