（資料7）ヒト ES 細胞由来混合コロニー中に含まれる赤血球における β グロビンの発現
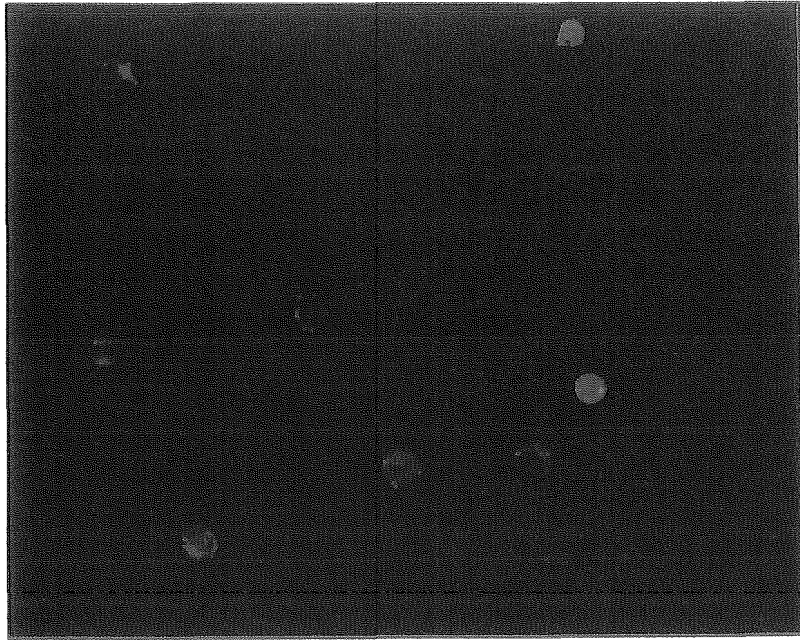
Ⅱ. 分担研究報告

厚生労働科学研究費補助金

（医薬品・医療機器等レギラトリーサイエンス総合研究事業）

分担研究報告書

ヒト胚性幹細胞の分化の分子生物学的解析

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研究要旨

胎生 14〜15 日のマウス胎仔肝由来ストローマ細胞には、少なくともヘパトサイトと内皮細胞が混在しており、それらの細胞の協同作用によりヒト胚性幹細胞（ES 細胞）は血液細胞に分化誘導されると推測された。今後、これらの細胞の characterization をすすめ、ヒト ES 細胞は血液細胞に分化誘導を担っている分子を同定することにより、異種動物のストローマに依存しない、ヒト ES 細胞から血液細胞への分化誘導法が確立されることが期待される。

A. 研究目的

　マウスの胎生期造血は、卵黄嚢に発生し、胎生初期造血を担う一次造血と、その後の一生にわたる造血を支えることになる二次造血にわけられる。二次造血は、マウス胎仔の胎生 10 日の AGM（Aorta-Gonad-Mesonephros）領域に発生し、その後、造血の場を胎仔肝に移し、爆発的に増幅した後、脾臓、骨髄へ移動し、それ以降の一生にわたる造血を担うことになる。我々は、マウスの胎生期の造血環境を再現することにより、同じ哺乳類であるヒトの胚性幹細胞（ES 細胞）の血液細胞への分化誘導が可能ではないかと考えた。

　その結果、主任研究者の研究成果より、ヒト ES 細胞は、胎生 14〜15 日のマウス胎仔肝由来ストローマ細胞と

検討を行った。
①形態学的観察
②免疫細胞学的検討
　α-fetoprotein（AFP）、アルブミン
　の発現の検討
③フローサイトメトリーによる検討
　Sca-1、CD13、CD31、CD34、CD49d、
　VE-カドヘリン（VE-Cad）の発現の検
　討

2.　胎仔肝以外のマウス胎生期造血
臓器（AGM 領域および骨髄）由来スト
ローマ細胞とマウス胎仔肝由来スト
ローマ細胞のヒト ES 細胞の分化に及
ぼす影響の比較検討
　　胎生 11～12 日のマウス胎仔の AGM
領域、あるいは、胎生 18～19 日のマ
ウス胎仔骨髄からストローマ細胞を
樹立し、ヒト ES 細胞と共培養する。
これらの共培養系における血液細胞
の産生について、胎生 14～15 日のマ
ウス胎仔肝由来ストローマ細胞との
共培養と比較する。

3.マウス胎仔肝由来ストローマ細胞
を継代し、cell line 化を試みる。

（倫理面への配慮）
本研究の遂行にあたっては、「ヒト ES
細胞の樹立および使用に関する指針」
を遵守して実施された。なお、本研究
の過程において、クローン胚が作製さ
れる危険性はない。
　　本研究計画は、東京大学バイオサイ
エンス委員会ヒト生殖・クローン専門
委員会の承認を得た後、平成 14 年 12
月 20 日に文部科学省特定胚及びヒト
ES 細胞研究専門委員会にて承認され
た。

C. 研究成果

1. マウス胎仔肝由来ストローマ細胞
の characterization

① 形態学的観察（資料 1）
　　胎生 14～15 日のマウス胎仔肝由来
のストローマ細胞は、形態学的には、
大型の扁平な細胞と、紡錘形の細胞
の、少なくとも 2 種類の細胞が存在
すると推測された。

② 免疫細胞学的検討（資料 2）
　　胎生 14～15 日のマウス胎仔肝由来
のストローマ細胞の一部は、AFP やア
ルブミンを持ったヘパトサイトであ
ると考えられた。

③ フローサイトメトリーによる検討
　（資料 3）
　　胎生 14～15 日のマウス胎仔肝由来
のストローマ細胞は、一部に Sca-1、
CD13、CD31、CD34、CD49d、VE-Cad な
どの内皮細胞のマーカーを発現して
いた。

2.　胎仔肝以外のマウス胎生期造血
臓器（AGM 領域および骨髄）由来スト
ローマ細胞とマウス胎仔肝由来スト
ローマ細胞のヒト ES 細胞の分化に及
ぼす影響の比較検討
　　胎生 11～12 日のマウス胎仔の AGM
領域、あるいは、胎生 18～19 日のマ
ウス胎仔骨髄からも、ストローマ細
胞の樹立は可能ではあったが、マウ
ス AGM 領域からストローマ細胞を樹
立することは、技術的に難しく、そ
の維持も容易ではなかった。また、
マウス AGM 領域から、大量のストロ
ーマ細胞を樹立することは困難であ
った。

ヒトES細胞を、マウス胎仔骨髄から樹立されたストローマ細胞と共培養しても、血液細胞への分化誘導はほとんど認められなかった。一方、AGM領域由来のストローマ細胞は、胎仔肝由来のストローマ細胞とほぼ同等の分化誘導能を有していた。

3. マウス胎仔肝由来ストローマ細胞のcell line化

胎生14〜15日のマウス胎仔肝由来のストローマ細胞を継代することにより、数株のcell lineを樹立することができたが、いずれのcell lineと共培養しても、ヒトES細胞は血液細胞を産生することはできなかった。

D. 考察

ヒトES細胞から血液細胞への分化誘導能を保持したまま、胎生14〜15日のマウス胎仔肝由来のストローマ細胞をcell line化できなかった理由としては、以下のようなことが考えられる。
1) ヒトES細胞から血液細胞への分化誘導を担うストローマ細胞は、胎生期の特定の時期にのみ出現するため、長期培養することができない。
2) マウス胎仔肝由来のストローマ細胞上のヒトES細胞から血液細胞への分化誘導を担う分子は、一過性にしか発現しないため、長期培養の間に消失してしまう。
3) ヒトES細胞から血液細胞への分化誘導は、単一の細胞ではなく、2種類以上の細胞による協同作用によるため、クローニングされたストローマ細胞単独では、ヒトES細胞を血液細胞へ分化誘導できない。
実際、胎生14〜15日のマウス胎仔

肝由来ストローマ細胞は、形態学的観察では、少なくとも2種類の細胞が混在していると推測された。また、細胞免疫学的解析やフローサイトメトリーによる検討では、ヘパトサイトと内皮細胞が存在することが確認された。

また、マウス胎仔AGM領域由来ストローマ細胞も、ヒトES細胞から血液細胞への分化誘導能という点では、マウス胎仔肝由来ストローマ細胞とほぼ同等の能力を有していたが、前者の樹立、維持は後者と比較して難しく、樹立されるストローマ細胞数も少ないため、大量のcell processingには、マウス胎仔肝ストローマ細胞の方がすぐれていると考えられた。

E. 結論

胎生14〜15日のマウス胎仔肝由来のストローマ細胞には、少なくともヘパトサイトと内皮細胞が混在しており、それらの細胞の協同作用によりヒトES細胞は血液細胞に分化誘導されると推測された。また、マウス胎仔肝由来ストローマ細胞は、AGM領域由来ストローマ細胞、胎仔骨髄由来細胞と比較して、ヒトES細胞から大量の血液を産生するのに適していると考えられた。

今後、さらにマウス胎仔肝由来ストローマ細胞のcharacerizationを進めることにより、ヒトES細胞から血液細胞への分化誘導を担っている分子を同定し、異種動物のストローマに依存しない、ヒトES細胞からの血液細胞産生法を確立したいと考えている。

F. 研究発表

1. 論文発表

RAS-blocking bisphosphonate zoledronic acid inhibits the abnormal proliferation and differentiation of juvenile myelomonocytic leukemia cells *in vitro*.
Ohtsuka Y, Manabe A, Kawasaki , H, Hasegawa D, Zaike Y, Watanabe S, Tanizawa T, Nakahata T, Tsuji K:
Blood 106: 3134-3141, 2005.

Methylation status of the *p15* and *p16* genes in paediatric myelodysplastic syndrome and juvenile myelomonocytic leukaemia.
Hasegawa D, Manabe A, Kubota T, Kawasaki H, Hirose I, Ohtsuka Y, Tsuruta T, Ebihara Y, Goto Y, Zhao XY, Sakashita K, Koike K, Isomura M, Kojima S, Hoshika A, Tsuji K, Nakahata T.
Br J Haematol 128: 805-812. 2005

2. 学会発表

A novel method for the efficient production of multipotential hematopoietic progenitors from human embryonic stem cells by co-culture with murine fetal liver-derived stromal cells.
Ma F, Wang D, Hanada S, Kawasaki H, Zaike Y, Heike T, Kitamura T, Nakahata T, Tsuji K:
International Symposium on Germ Cells, Reprogramming and Embryonic Stem Cells.
Kyoto, Nov 10-13, 2005.

A novel method for the efficient production of multipotential hematopoietic progenitors from human embryonic stem cells by co-culture with murine fetal liver-derived stromal cells.
Ma F, Wang D, Hanada S, Kawasaki H, Zaike Y, Heike T, Kitamura T, Nakahata T, Tsuji K:
47th Annual Meeting, American Society of Hematology,
Atlanta, December 10-13, 2005.

非血縁者間同種骨髄移植後の再発に対するドナーリンパ球輸注（DLI）の臨床検討
河崎裕英、長谷川大輔、大塚欣敏、鶴田敏久、真部淳、海老原康博、辻浩一郎：
第108回日本小児科学会学術集会
東京、2005年4月22-24日

リンパ腫様肉芽腫症の1例
河崎裕英、内丸薫、高橋聡、東條有伸、辻浩一郎：
第47回日本臨床血液学会総会
横浜、2005年9月17-19日

JMML における赤芽球系コロニー形成
能の検討
長谷川大輔、真部淳、石川久実子、和
田美夏、谷ヶ崎博、吉益哲、河崎裕英、
海老原康博、中畑龍俊、辻浩一郎、
第47回日本臨床血液学会総会
横浜、2005年9月17-19日

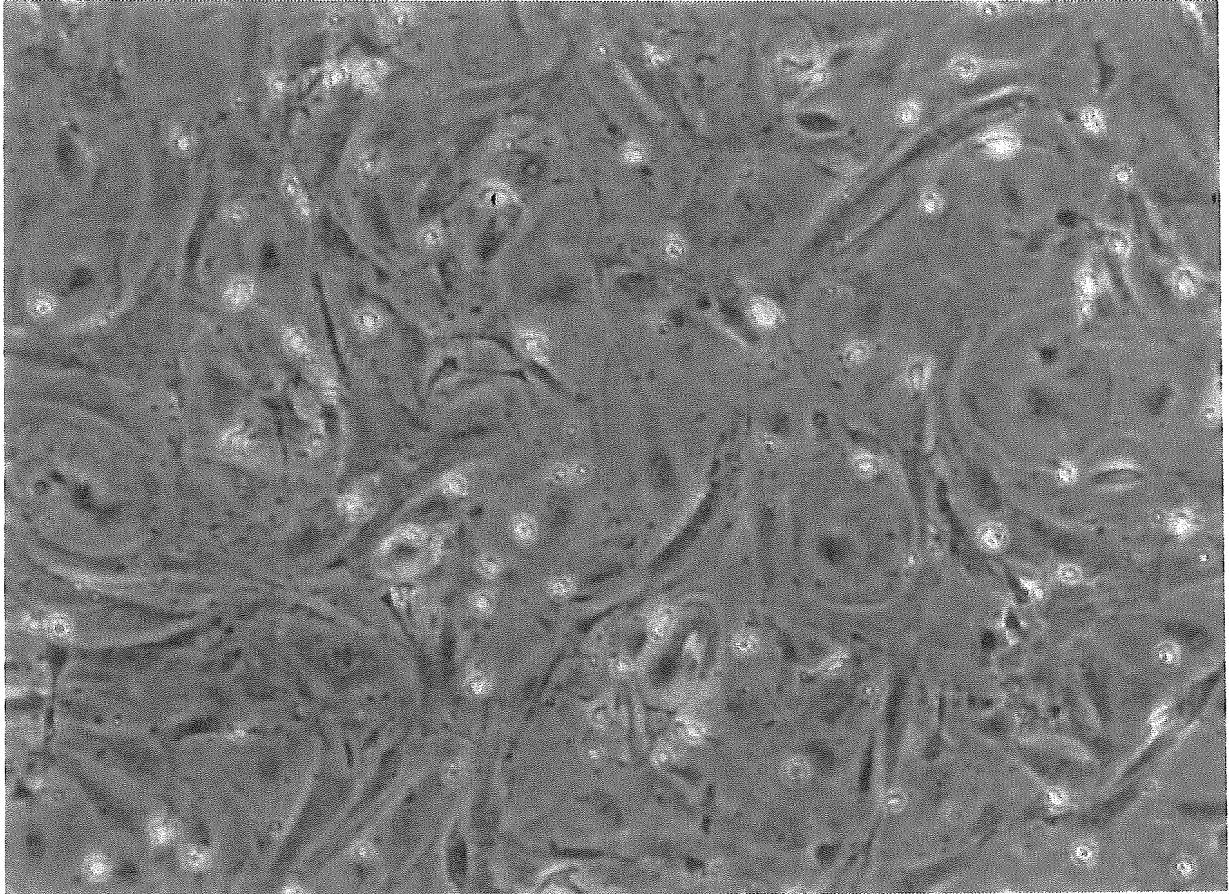若年性骨髄単球性白血病（JMML)にお
ける赤芽球系コロニー形成に対する
ビスフォスフォネート製剤の効果
大塚欣敏、浅野由美、金田由美、竹田
洋樹、森田直子、長谷川大輔、河崎裕
英、真部淳、辻浩一郎、谷澤隆邦：
第47回日本臨床血液学会総会
横浜、2005年9月17-19日

小児におけるリンパ腫様肉芽腫症
河崎裕英、辻浩一郎：
第47回日本小児血液学会総会
宇都宮、2005年11月25-27日

G. 知的財産権の出願・登録状況（予定
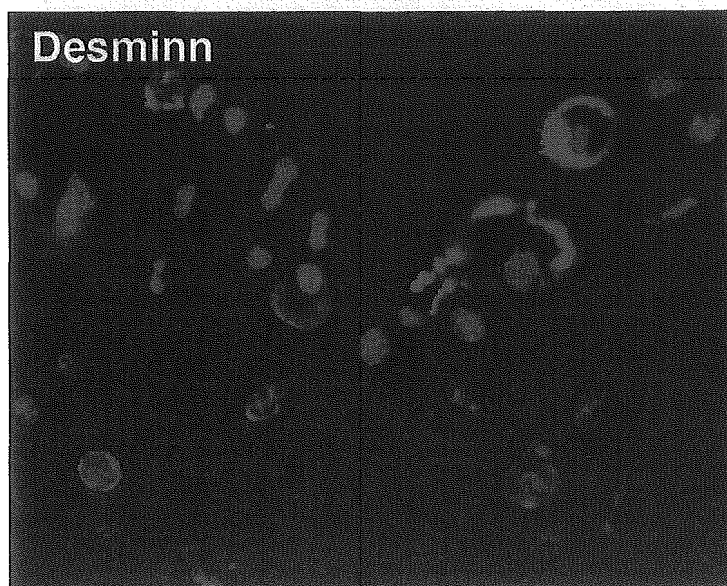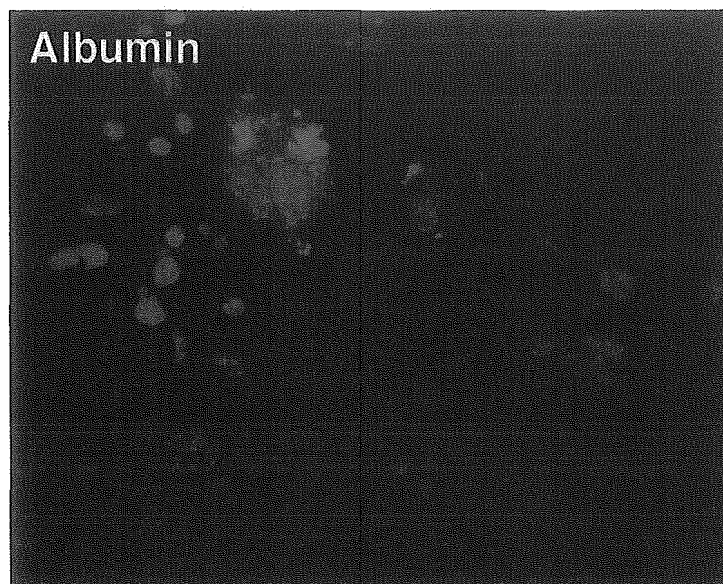　を含む）

1. 特許取得
　該当なし。

2. 実用新案登録
　該当なし。

3. その他
　該当なし。

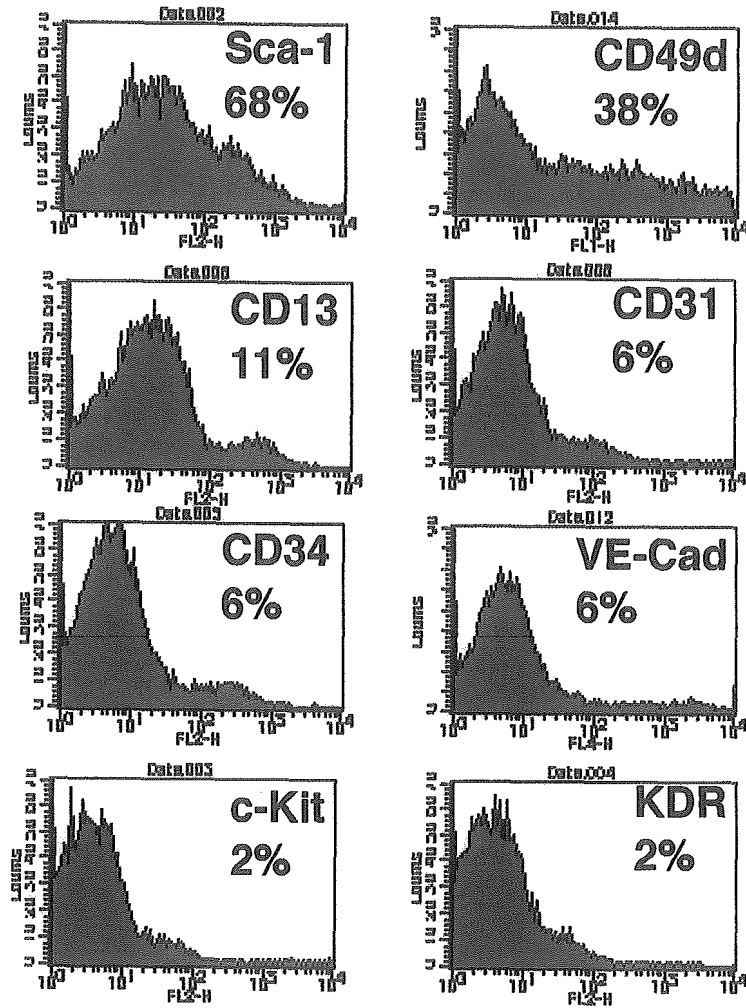（資料１）マウス胎仔肝由来ストローマ細胞

（資料２）マウス胎仔肝由来ストローマ細胞における Albumin および Desminn
の発現

（資料３）マウス胎仔肝由来ストローマ細胞のフローサイトメトリーによる解析

Ⅲ. 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
辻浩一郎	遺伝子治療	別所文雄 横森欣司	よく理解できる子どものがん—診療から看護ケアまで—	永井書店	東京	印刷中	
辻浩一郎	臍帯血と胎生期造血	浅野茂隆 谷口克 中畑龍俊	臍帯血移植の基礎と臨床	医学書院	東京	印刷中	
辻浩一郎	幹細胞をめぐる現状と展望	工藤翔二 土屋了介 金沢実 大田健	Annual Review 呼吸器 2007	中外医学社	東京	印刷中	

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
Hasegawa D, Manabe A, Kubota T, Kawasaki H, Hirose I, Ohtsuka Y, Tsuruta T, Ebihara Y, Goto Y, Zhao XY, Sakashita K, Koike K, Isomura M, Kojima S, Hoshika A, Tsuji K, Nakahata T	Methylation status of the p15 and p16 genes in paediatric myelodysplastic syndrome and juvenile myelomonocytic leukaemia.	Br J Haematol	128	805-812	2005
Ohtsuka Y, Manabe A, Kawasaki H, Hasegawa D, Zaike Y, Watanabe S, Tanizawa T, Nakahata T, Tsuji K	RAS-blocking bisphosphonate zoledronic acid inhibits the abnormal proliferation and differentiation of juvenile myelomonocytic leukemia cells *in vitro*.	Blood	106	3134-3141	2005
Sugiyama D, Arai K, Tsuji K	Definitive hematopoiesis from acetyl LDL incorporating endothelial cells in the mouse embryo.	Stem Cells and Development	14	687-696	2005

44

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, Tsuji K	Definitive hematopoiesis from endothelial cells in mouse embryo: A simple guidance.	Trends Cardiovas Med	In press		
Ma F, Wang D, Hanada S, Ebihara Y, Kawasaki H, Zaike Y, Heike T, Nakahata T, Tsuji K	Novel method for efficient production of multipotential hematopoietic progenitors from human embryonic stem cells.	Blood	In press		
辻浩一郎	造血幹細胞.	最新医学	60	1695-1700	2005
辻浩一郎	ヒト胚性幹細胞からの血液産生と再生医療.	日小血会誌	19	175-180	2005
辻浩一郎	JMML に対するビスフォスフォネートの効果.	血液・腫瘍科	印刷中		
辻浩一郎	Embryonic stem cell の造血幹細胞移植への応用	今日の移植	印刷中		

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
辻浩一郎	ヒト ES 細胞からの血液細胞の産生	臨床血液	印刷中		
辻浩一郎	末梢血幹細胞	血液・腫瘍科	印刷中		
辻浩一郎	がんの細胞療法	日本アフェレシス学会雑誌	印刷中		

IV. 研究成果の刊行物・別冊

RAS-blocking bisphosphonate zoledronic acid inhibits the abnormal proliferation and differentiation of juvenile myelomonocytic leukemia cells in vitro

Yoshitoshi Ohtsuka, Atsushi Manabe, Hirohide Kawasaki, Daisuke Hasegawa, Yuji Zaike, Sumiko Watanabe, Takakuni Tanizawa, Tatsutoshi Nakahata, and Kohichiro Tsuji

Juvenile myelomonocytic leukemia (JMML) is a clonal myeloproliferative/myelodysplastic disorder of early childhood with a poor prognosis. JMML cells are characterized by hypersensitivity to granulocyte-macrophage colony-stimulating factor (GM-CSF) caused by a continuously activated GM-CSF receptor–retrovirus-associated sequence (RAS) signal transduction pathway through various molecular mechanisms, resulting in spontaneous GM colony formation in vitro. Bisphosphonate zoledronic acid (ZOL), a RAS-blocking compound, suppressed colony formation from bone marrow (BM) cells of 8 patients with JMML and 5 healthy control subjects without and with GM-CSF (10 ng/mL), respectively, in a dose-dependent manner in clonal culture. At 10 μM ZOL, however, spontaneous GM colony formation from JMML BM cells decreased to 3%, but the formation of G colonies containing granulocytes, but no macrophages, was enhanced, whereas 40% of GM colonies were retained and G colony formation was not affected in culture of normal BM cells with GM-CSF. In suspension culture, cytochemical and flow cytometric analyses showed that 10 μM ZOL also inhibited spontaneous proliferation and differentiation along monocyte/macrophage lineage of JMML BM cells but not the development of normal BM cells by GM-CSF. The inhibitory effect of ZOL on JMML cells was confirmed at a single-clone level and observed even at 3 μM. The current result offers a novel approach to therapy in JMML. (Blood. 2005; 106:3134-3141)

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Introduction

Juvenile myelomonocytic leukemia (JMML) is a rare clonal myeloproliferative/myelodysplastic disorder of infancy and early childhood.[1-3] Despite various approaches to therapy, the mortality rate in patients with JMML is still high. Intensive chemotherapeutic regimens have largely proved futile in inducing durable remissions.[4] Low-dose chemotherapy with 6-mercaptopurine, for example, has been temporarily effective in some patients, but generally it has not been shown to result in long-term disease control.[5,6] Allogeneic hematopoietic stem cell transplantation (SCT) is presently the only therapy capable of producing durable remissions, but the 4- or 5-year probability of event-free survival in patients with JMML treated by SCT is approximately 50%.[5,7] Therefore, the development of a new treatment for patients with JMML is awaited.

JMML cells are characterized by the ability to spontaneously proliferate in the absence of hematopoietic growth factors in vitro,[8] giving rise to granulocyte-macrophage (GM) colonies caused by hypersensitivity to granulocyte-macrophage colony-stimulating factor (GM-CSF) released from monocytes/macrophages.[9] Several investigators have shown that deregulated GM-CSF signal transduction through the retrovirus-associated sequence (RAS) pathway plays a key role in the characteristic property of JMML cells.

Activating mutations of the RAS gene were found in 15% to 30% of patients with JMML.[10-14] It was also reported that 30% of patients with JMML had mutations of the gene NF1 encoding neurofibromin, a guanosine triphosphatase acting protein that inactivates RAS, leading to a continuously activated GM-CSF receptor (GM-CSFR)–RAS pathway.[15] Furthermore, somatic mutations of PTPN11 encoding a cytoplasmic Src homology-2 domain containing protein that controls RAS functions were found in 34% of patients with JMML without Noonan syndrome.[16] Given these reports, linking the pathogenesis of JMML to the continuously activated GM-CSFR–RAS signal transduction pathway, it is reasonable to explore molecular mechanism-based therapy for JMML.

A GM-CSF antagonist, E21R, was earlier shown to inhibit JMML-cell growth in vitro and JMML-cell engraftment in immunodeficient mice.[17,18] Another feasible way to inhibit JMML-cell development is to block the GM-CSFR–RAS signaling intracellular pathway. The first obligatory step in the signal transduction, which is essential for RAS activity, is the membrane localization of RAS accomplished through a prenylation reaction mediated by farnesyltransferase (FTase), which involves the covalent linking of a 15-carbon isoprenyl (farnesyl) group to RAS.[19-21] FTase inhibitors (FTIs) have been evaluated in several in vitro and in vivo

preclinical systems and demonstrated some antitumor effects.[22,23] However, even when FTase is inhibited, RAS can be transferred to the membrane by an alternative pathway using geranylgeranyl transferase-1.[24] Bisphosphonate (BP) has been used to treat bone diseases and acted as an anticancer drug by inhibiting the activation of RAS through the suppression of both farnesylation and geranylgeranylation.[25,26] More recently, zoledronic acid (ZOL), a third-generation BP, has been shown to be more effective in blocking RAS activity.[27] We then investigated the effect of ZOL on JMML-cell growth in vitro to clarify the function of continuously activated RAS in JMML cells and to evaluate the feasibility of using ZOL as an antileukemia drug for JMML.

Materials and methods

Zoledronic acid

Zoledronic acid (2-(imidazol-1-yl)-hydroxy-ethylidene-1, 1-bisphosphonic acid, disodium salt, 4.75 hydrate) was supplied by Novartis Pharma AG (Basel, Switzerland), dissolved in a stock solution of water at a concentration of 100 mM and stored at $-20°C$ in plastic (not glass) containers.

Acquisition of donor samples

With personal and parental consent, bone marrow (BM) samples were obtained from children with JMML diagnosed based on the criteria of the International JMML Working Group.[28] Normal controls were healthy adult volunteers who donated BM samples after providing informed consent. The study protocol was approved by the MDS Committee of the Japanese Society of Pediatric Hematology. Plastic syringes (10 mL) coated with preservative-free heparin were used for the acquisition of BM samples. Nonphagocytic mononuclear cells (NPMNCs) were separated by Ficoll-Hypaque density centrifugation after the depletion of phagocytes with Silica (Immuno Biological Laboratories, Fujioka, Japan). The NPMNCs were washed twice and suspended in α-medium (Flow Laboratories, Rockville, MD). Nonerythroid cells were obtained by lysis with NH_4CL.

Clonal culture

The isolated BM NPMNCs were incubated in methylcellulose culture in triplicate using a technique described previously[29] with some modifications. Briefly, 1 mL culture mixture containing 2×10^4 cells, α-medium, 0.9% methylcellulose (Shinetsu Chemical, Tokyo, Japan), 30% fetal bovine serum (FBS; Hyclone, Logan, UT), 1% deionized fraction V bovine serum albumin (BSA; Sigma, Saint Louis, MO), 5×10^{-5} M mercaptoethanol, and various concentrations of ZOL was plated in each 35-mm standard nontissue culture dish (Nunc, Roskilde, Denmark) and incubated at 37°C in a humidified atmosphere flushed with 5% CO_2 in air. In some experiments, GM-CSF (Stem Cell Technologies, Vancouver, Canada) was added to the culture mixture at a concentration of 10 ng/mL that induced an optimal response in methylcellulose culture of human BM hematopoietic cells.[30] The size and type of colonies (> 40 cells) formed in the culture were assessed at day 14 of culture. The size of small colonies was determined by direct cell counting in situ under an inverted microscope. When colonies contained more than 200 cells, they were removed individually with an Eppendorf micropipette and prepared as single-cell suspensions. Colony size was estimated using a counting chamber with the cell suspension. Colony types were determined according to criteria reported previously[31]: granulocyte (G), monocyte/macrophage (M), and GM colonies that consist of granulocytes, including neutrophils, eosinophils, and basophils, monocytes/macrophages, and both granulocytes and monocytes/macrophages, respectively. To assess the accuracy of the in situ identification for the colony types, individual colonies were lifted, spread on glass slides using a cytocentrifuge (Cytospin 2; Shandon Southern Instruments, Sewickley, PA), and treated with May-Grünwald-Giemsa staining and cytochemical staining with α-naphthyl butyrate esterase (α-NBE) according to conven-

tional methods. Differential counts of the cells were done with more than 100 cells on the cytospin smears in all experiments.

Suspension culture

Twenty thousand nonerythroid BM cells were incubated in a suspension culture using a modified version of a technique described previously.[32,33] Briefly, 1 mL culture mixture containing 5×10^4 cells, α-medium, 30% FBS, 1% deionized fraction V BSA, and various concentrations of ZOL was incubated in 12-well tissue plates (Nunc) at 37°C in a humidified atmosphere flushed with 5% CO_2 in air. The number of cultured cells and the cellular composition were determined at days 0, 5, and 10. The cell number was assessed by direct cell counting of liquid culture aliquots in a hemocytometer. The cellular composition was determined on cytospin smears of the cultured cells stained and by flow cytometric analysis (see "Flow cytometry"). In the special experiment, colonies developed in clonal culture at day 5 were individually collected in a microtube containing 100 μL α-medium and then divided into 2 aliquots. Each half was incubated in a suspension culture with or without 10 μM ZOL for 10 days after the addition of 50 μL respective culture medium.

Flow cytometry

Flow cytometric analysis was carried out using myelomonocytic cell-differentiation–related antigens; fluorescein isothiocyanate (FITC)–conjugated CD14, CD16, and human leukocyte antigen (HLA)–DR; phycoerythrin (PE)–conjugated CD11b and CD13; and peridininchlorophyll (PerCP)–conjugated CD45. All monoclonal antibodies used were purchased from Becton Dickinson Immunocytometry Systems (San Jose, CA). The staining procedure was described previously.[34] All incubations with antibodies were carried out for 30 minutes on ice. Flow cytometric data were acquired on a fluorescence-activated cell scan (Becton Dickinson) using the Lysis-II software program. In a standard 3-color immunofluorescence protocol, forward and side scatters (FSC and SSC, respectively) were collected along with 3-color antibody combinations. Electronic gating on the basis of FSC and SSC excluded cellular debris and nonviable cells. Cell population percentages of monocytes/macrophages, granulocytes, lymphocytes, and blastic cells in nonerythroid cells were assessed by SSC and CD45 staining as described.[35] The flow cytometric profile was obtained by either directly gating discrete populations identified by SSC and PerCP-conjugated CD45 staining, or by back-gating using FITC- and PE-conjugated antibody combinations to identify cells of interest.

Statistical analysis

Student t test was used to determine the significance of differences among groups of unpaired samples in all experiments. P values were derived from 2-sided tests, and P less than .05 was considered statistically significant.

Results

Effect of ZOL on colony growth from JMML and normal BM cells in clonal culture

Eight children with JMML were enrolled in this study (6 boys and 2 girls). Their median age was 3 years and 1 month (range, 1 month to 5 years and 9 months).

In clonal culture, BM cells of all 8 patients produced a large number of spontaneous colonies in the absence of hematopoietic factors, and the number of colonies was comparable to that formed in the presence of 10 ng/mL GM-CSF in 6 of 8 patients (Table 1). In cases 3 and 6, however, the number of colonies increased with the addition of 10 ng/mL GM-CSF to the culture. Most colonies formed in the absence or presence of 10 ng/mL GM-CSF were GM colonies that consisted of both granulocytes and macrophages

Table 1. Colony formation from BM cells of patients with JMML and healthy adults

Case no.	No. of colonies formed, 2 × 10⁴ BM NPMNCs		GM colonies in total colonies, %	
	GM-CSF absent	GM-CSF present*	GM-CSF absent	GM-CSF present*
Patients with JMML				
1	135 ± 23	160 ± 14	97	99
2	153 ± 33	190 ± 22	100	100
3	44 ± 12	121 ± 13	98	100
4	193 ± 34	168 ± 33	97	99
5	131 ± 32	177 ± 32	95	99
6	46 ± 11	94 ± 21	100	100
7	152 ± 21	206 ± 16	96	99
8	217 ± 31	198 ± 22	99	98
Mean ± SD	137 ± 41	160 ± 36	98 ± 2	99 ± 0.2
Healthy adults				
1	0 ± 0	155 ± 22	NA	93
2	0 ± 0	28 ± 13	NA	65
3	0 ± 0	234 ± 35	NA	84
4	0 ± 0	104 ± 21	NA	91
5	0 ± 0	200 ± 22	NA	95
6	0 ± 0	203 ± 19	NA	97
Mean ± SD	0 ± 0	154 ± 23	NA	87 ± 11

NA indicates not applicable.
*GM-CSF was used at a concentration of 10 ng/mL, which induced an optimal response among normal BM cells.

(Table 1; Figure 1A). By contrast, BM cells of healthy adults produced no colonies in the absence of hematopoietic factors. On the addition of GM-CSF, normal BM cells produced colonies. Eighty-seven percent of the colonies were GM colonies whose appearance revealed a diminished number of macrophages (Figure 1B) as compared with spontaneous GM colonies generated from JMML BM cells, and the rest were G colonies (12% ± 11%, n = 6) with only a few M colonies (< 1%).

When added to the culture, ZOL suppressed the spontaneous colony formation from BM cells of all 8 patients with JMML in a dose-dependent manner (Figure 2A). The number of spontaneous colonies formed from JMML BM cells decreased to a 3rd and a 15th at concentrations of 10 and 100 µM ZOL, respectively. In particular, the formation of GM colonies was much decreased at 10 µM ZOL (3% ± 3%), and no GM colonies were produced at 100 µM ZOL. Furthermore, the size of spontaneous colonies was smaller at greater than 10 µM ZOL. As shown in Figure 2B, the numbers of constituent cells in individual spontaneous colonies (n = 15 at each concentration of ZOL) randomly chosen from the cultures of BM cells of 3 patients with JMML (cases 4, 6, and 7) with 10 and 100 µM ZOL were much smaller than those with no or

1 µM ZOL. Interestingly, the addition of ZOL enhanced the formation of small G colonies consisting of only granulocytes, with no macrophages (Figures 1C and 2A). The effect of ZOL on the formation of colonies from BM cells of patients with JMML (cases 4 and 7) in the clonal culture containing 10 ng/mL GM-CSF was also examined. We again observed that 10 µM ZOL inhibited GM colony formation and enhanced G colony formation in a manner similar to that observed for the spontaneous colony formation (data not shown).

However, when 10 and 100 µM ZOL were added to the culture of normal BM cells with 10 ng/mL GM-CSF, the number of colonies decreased to approximately 60% and 20%, respectively (Figure 2A), but at 10 µM ZOL, GM colonies still occupied the larger proportion of the total colonies retained (69% ± 14%, significantly different from the percentage [8% ± 7%] in spontaneous GM colony formation by JMML BM cells [$P < .01$]). ZOL did not have a significant effect on the formation of G colonies from normal BM cells in the presence of 10 ng/mL GM-CSF. The size of the GM colonies also decreased in a dose-dependent manner, but it was much larger than that of the spontaneous colonies from JMML samples at 10 µM ZOL ($P < .01$) (Figure 2B).
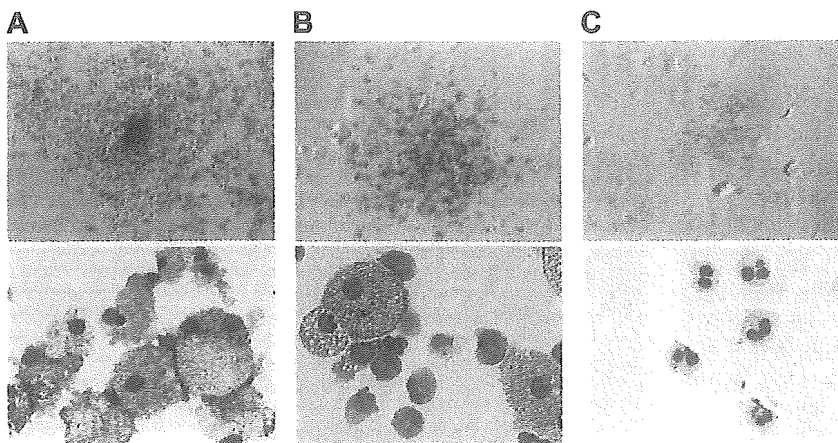
A   B   C

Figure 1. Colonies formed under various conditions. Top and bottom panels show the appearance of colonies under an inverted microscope (ITM; Olympus, Tokyo, Japan) equipped with a camera module (DP50; Olympus) (objective lens, SPlan 4PL; numerical aperture, 0.13; magnification, × 4) and their constituent cells stained with May-Grünwald-Giemsa solution (microscope, BX51, Olympus; camera module, XC-003, Sony, Tokyo, Japan; objective lens, UPlan F1, Olympus; numerical aperture, 1.3; magnification, × 100), respectively. (A) A spontaneous GM colony in the culture of JMML BM cells without hematopoietic factors. (B) A GM colony in the culture of normal BM cells with 10 ng/mL GM-CSF. (C) A spontaneous G colony in the culture of JMML BM cells with 10 µM ZOL.
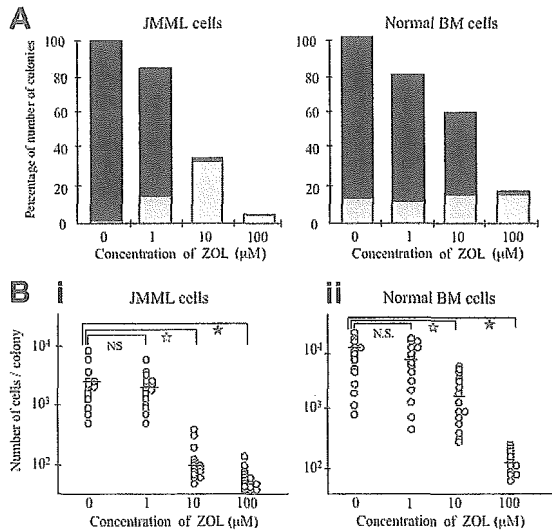
Figure 2. Effect of ZOL on colony formation from JMML and normal BM cells.
(A) The percentages of the number of colonies in the culture of JMML and normal BM cells without and with 10 ng/mL GM-CSF, respectively, in the presence of various concentrations of ZOL to that in the absence of ZOL, and the proportion of G and GM colonies in total colonies under the respective conditions. The values indicate the means calculated from the data for 8 patients with JMML and 6 healthy donors in Table 1. ■ indicates GM colonies; ▨, G colonies. (B) The numbers of cells contained in individual colonies cultured from JMML and normal BM cells without and with 10 ng/mL GM-CSF, respectively. Fifteen colonies were randomly chosen in the culture of BM cells of 3 patients with JMML and 3 healthy donors under the respective conditions. Bars indicate the means of cell numbers of the 15 colonies. In i, NS indicates not significant; ✬, $P < .01$; ✯, $P < .001$. In ii, ✬ indicates $P < .05$; ✯, $P < .001$.

Effect of ZOL on development of JMML and normal BM cells in suspension culture

The result of the clonal culture suggested that ZOL inhibited the proliferation and differentiation of both JMML and normal BM cells, but the inhibitory effect was stronger in the former. We then carried out suspension culture to examine the effect of ZOL on the development of BM cells of patients with JMML and healthy donors in more detail.

In suspension culture without hematopoietic factors, nonerythroid BM cells of 3 patients with JMML (cases 4, 6, and 7) proliferated, whereas no increase of the cells cultured from BM cells of 3 healthy donors (cases 1, 3, and 5) was observed. With the addition of 10 ng/mL GM-CSF to the culture, normal BM cells proliferated. When incubated for 15 days, both JMML and normal BM cells showed the development of a number of adherent cells in the suspension culture without and with 10 ng/mL GM-CSF, respectively, as shown in Figure 3A. Therefore, we could not accurately calculate the number of cells contained in the cultures at day 15. When ZOL was added, no development of adherent cells was observed at greater than 10 μM in the culture of JMML BM cells at day 15. In the culture of normal BM cells, however, adherent cells developed at 10 μM ZOL in the presence of 10 ng/mL GM-CSF although they did not develop at 100 μM.

Figure 3B shows the effect of ZOL on the proliferation of BM cells of 3 patients with JMML (cases 4, 6, and 7) and 3 healthy donors (cases 1, 3, and 5) in the suspension culture. Each value represents the mean ± SD of the percentages of the cells cultured at the respective concentrations of ZOL to those without ZOL at days 5 and 10 of culture. At day 5, 100 μM, but neither 1 nor 10 μM, ZOL inhibited the proliferation of JMML BM cells, but at day 10, 1 to 100 μM ZOL inhibited it. However, 1 and 10 μM ZOL revealed no

inhibitory effect on the proliferation of normal BM cells even at day 10 of culture, whereas 100 μM ZOL inhibited it at days 5 and 10.

Figure 4A shows the effect of ZOL on the proportion of α-NBE–positive macrophages and –negative cells in the suspension culture of JMML and normal BM cells. JMML BM cells contained 34% ± 6% of monocytes/macrophages whose α-NBE–positive granules were fine as compared with those in normal BM monocytes/macrophages, and α-NBE–negative cells included granulocytes, lymphocytes, immature blastic cells, and nucleated erythroid cells. In the culture of JMML BM cells without ZOL and with 1 μM ZOL, most of the cells generated were α-NBE–positive macrophages at day 10, and α-NBE–negative populations contained a few granulocytes and immature blastic cells (Figure 4A-B). At 10 and 100 μM ZOL, however, the proportion of α-NBE–positive macrophages significantly decreased and that of α-NBE–negative cells increased. The majority of the α-NBE–negative cells were granulocytes, but there was a substantial number of blastic cells.

However, a large proportion of normal BM cells were α-NBE–negative granulocytes. The α-NBE–negative cells also included nucleated erythroid cells, lymphocytes, and a few blastic cells. In the culture of normal BM cells with 10 ng/mL GM-CSF, α-NBE–negative granulocytes accounted for three quarters of the cells generated, and the other quarter were α-NBE–positive macrophages (Figure 4A-B). The addition of ZOL did not affect the
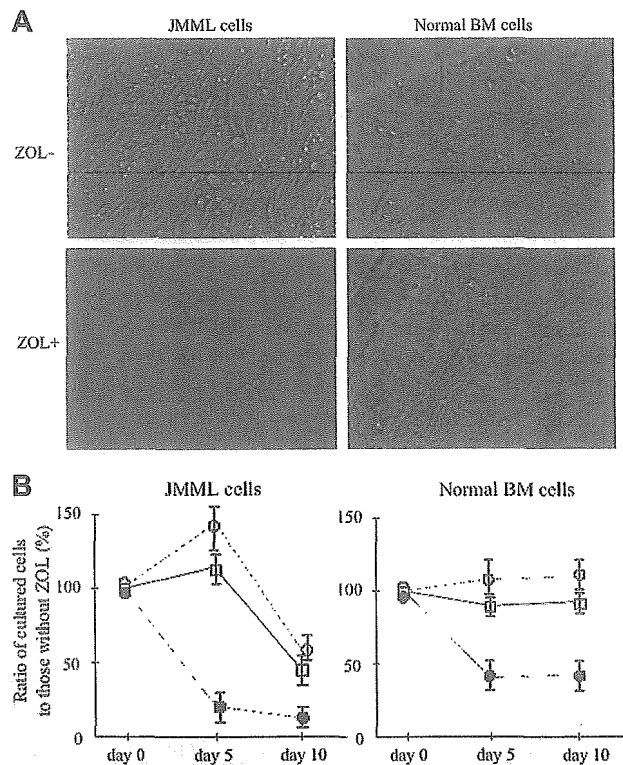

Figure 3. Effect of ZOL on the proliferation of JMML and normal BM cells in the suspension culture. (A) Phase microscopy of adherent cells developed from BM cells of a patient with JMML (case 4) and a healthy donor (case 3) at day 15 of suspension culture without and with 10 ng/mL ZOL (microscope, ITM2; camera module, DP50; objective lens, DPlan Ap10 UV, Olympus; numerical aperture, 0.4; magnification, × 40). A number of adherent cells were observed in the culture of JMML cells without ZOL, but their development was suppressed by the addition of ZOL. The growth of adherent cells in the culture of normal BM cells was not affected by the addition of ZOL. (B) The inhibition rate by 1, 10, and 100 μM ZOL in the proliferation of JMML and normal BM cells in the suspension culture without and with 10 ng/mL GM-CSF, respectively, at days 5 and 10. Each value indicates the mean ± SD calculated from the data in 3 patients with JMML and 3 healthy donors. □ indicates ZOL 1 μM; ○, ZOL 10 μM; ●, ZOL 100 μM.