レーショナルリサーチの安全性と信頼性が高まり，ひいては治療を受ける患者にも安全・安心な治療を提供できるものと信じる．

文献

1) 前川　平：厚生労働科学研究費補助金研究報告書，先端医療センター等における細胞治療・再生治療開発のための GMP 準拠細胞プロセッシング指針の作成に関する研究．2002．
2) ヒューマンサイエンス振興財団：規制動向調査報告書，レギュラトリーサイエンス—現状と課題．2004．
3) 厚生労働省医薬食品局血液対策課：平成 17 年度版血液事業報告．2005．
4) ヒト又は動物細胞株を用いて製造されるバイオテクノロジー応用医薬品のウイルス安全性評価について(ICHQ5 A)，(平成 12 年医薬審第 329 号)，2000．
5) 日本薬局方：参考情報 18．日局生物薬品のウイルス安全性確保の基本要件(平成 14 年厚生労働省告示第 395 号)．2002．
6) 国立感染症研究所：検定・検査業務必携．2006．

＊　　　＊　　　＊

Loss of Tie2 receptor compromises embryonic stem cell–derived endothelial but not hematopoietic cell survival

Isao Hamaguchi, Tohru Morisada, Masaki Azuma, Kyoko Murakami, Madoka Kuramitsu, Takuo Mizukami, Kazuyuki Ohbo, Kazunari Yamaguchi, Yuichi Oike, Daniel J. Dumont, and Toshio Suda

Tie2 is a receptor-type tyrosine kinase expressed on hematopoietic stem cells and endothelial cells. We used cultured embryonic stem (ES) cells to determine the function of Tie2 during early vascular development and hematopoiesis. Upon differentiation, the ES cell–derived $Tie2^+Flk1^+$ fraction was enriched for hematopoietic and endothelial progenitor cells. To investigate lymphatic differentiation, we used a monoclonal antibody against LYVE-1 and found that $LYVE-1^+$ cells derived from $Tie2^+Flk1^+$ cells possessed various characteristics of lymphatic endothelial cells. To determine whether Tie2 played a role in this process, we analyzed differentiation of $Tie2^{-/-}$ ES cells. Although the initial numbers of $LYVE-1^+$ and $PECAM-1^+$ cells derived from $Tie2^{-/-}$ cells did not vary significantly, the number of both decreased dramatically upon extended culturing. Such decreases were rescued by treatment with a caspase inhibitor, suggesting that reductions were due to apoptosis as a consequence of a lack of Tie2 signaling. Interestingly, $Tie2^{-/-}$ ES cells did not show measurable defects in development of the hematopoietic system, suggesting that Tie2 is not essential for hematopoietic cell development. (Blood. 2006;107:1207-1213)

© 2006 by The American Society of Hematology

Introduction

A close cell lineage relationship between hematopoietic and endothelial cells has long been recognized.[1,2] During embryogenesis, both cell types emerge in the yolk sac, and primitive erythrocytes differentiate juxtaposed to endothelial precursors by embryonic day 7.5 (E7.5).[3] In the mouse embryo, Tie2^{+} cells in the aorta-gonad-mesonephros (AGM) region generate both blood and endothelial cells.[4] From studies of embryonic stem (ES) cell differentiation, vascular endothelial growth factor (VEGF)–responsive bipotent precursors of hematopoietic and endothelial cells, known as hemangioblasts, have been identified.[5] Hemangioblast-derived endothelial cells form vascular vessels through vasculogenesis and angiogenesis. Lymphatic development starts when a subset of vascular endothelial cells of the cardinal vein commit to a lymphatic lineage and sprout to form the primary lymph sacs at around E9 or 10.[6,7] Mouse molecular genetic experiments indicate that Prox-1 (a homeobox transcription factor) and the VEGF receptor 3 (VEGFR-3) are crucial for the commitment of endothelial cells to a lymphatic lineage.[8-10] Since lymphatic vessel–specific molecules are being identified, the molecular mechanisms underlying development of lymphatic cells as well as vascular and hematopoietic cells can now be analyzed.

Many studies report that expression of Flk1 is crucial for early establishment of endothelial and hematopoietic lineages and perhaps for their common progenitor.[5,11,12] Flk1 encodes a receptor tyrosine kinase for the vascular endothelial growth factor family of ligands.[13] Single Flk1^{+} cells from embryoid bodies can give rise to blast colonies (blast lymphocyte colony-forming cells [BL-CFCs]), which produce both hematopoietic and endothelial cells in vitro.[5] Loss of Flk1 in mice results in selective defects in generating both blood and blood-vessel endothelial cells (BECs).[11] In addition to Flk1, Flt1 and Tie2 tyrosine kinases are also expressed in immature hematopoietic cells and BECs.[14-17]

The expression pattern of Tie2 suggests a function in both vascular endothelial and hematopoietic cells. Recently we determined the function of Ang1/Tie2 signaling, which maintains long-term repopulating hematopoietic stem cells in the bone marrow niche, suggesting that Tie2 signaling is crucial for adult bone marrow hematopoiesis.[18] In the mouse vitelline artery at E9.5, Tie2^{+} hematopoietic cells aggregate and adhere to endothelial cells.[19] In vitro culture of Tie2^{+} cells isolated from the AGM region generates both blood and endothelial cells.[4] In fetal liver, the Tie2^{-} fraction contains an enriched fraction of long-term repopulating cells.[20] Based on these findings, Tie2 signaling was thought to regulate embryonic development and differentiation of hematopoietic cells. However, more recently, Puri and Berstein have demonstrated that Tie2 is dispensable for embryonic hematopoiesis using ES cell mouse chimeras.[21] This finding suggests that Tie2 function in developmental hematopoiesis differs from its role in bone marrow hematopoiesis.

By contrast, Tie2 function in BECs has been intensively analyzed at both developmental and adult stages. In vivo and in vitro experiments show that Tie2 signaling potently induces sprouting, chemotaxis, and network formation.[22,23] Furthermore, the Tie2 ligand Ang1 is a potent survival factor for BECs under serum deprivation.[24] A role for Tie2 signaling in blood vessel endothelial survival in vivo has also been illustrated using conditional rescue of $Tie2^{-/-}$ embryos,[25] further supporting a role of this signaling system in endothelial cell survival.

Recently it has been demonstrated in mice that Tie2 is expressed and functions in lymphatic vessels embryonically[26] and in adults.[27] Tie2-deficient mice exhibit severe defects in vascular and heart development and die by E9.5,[28,29] making analysis of the lymphatic system difficult. Therefore, here we analyzed the function of Tie2 in lymphatic endothelial cells (LECs) and hematopoietic cells as well as in developing BECs using differentiation of cultured ES cells. We identify ES cell–derived LECs as well as BECs and hematopoietic cells, and demonstrate that Tie2 signaling is essential for development of BECs and LECs, but not for hematopoietic cells.

Materials and methods

Cell preparation and culture conditions

TT2,[30] E14,[31] and R1[32] ES cells were maintained on mouse embryonic fibroblast (MEF) feeder cell layers in knockout Dulbecco-modified Eagle medium (Gibco BRL, Carlsbad, CA) containing 15% fetal bovine serum (Intergen, Purchase, NY), 100 U/mL leukemia inhibitory factor (LIF; Chemicon International, Temecula, CA), 0.1 mM nonessential amino acids (Gibco BRL), 1 mM sodium pyruvate (Gibco BRL), 2 mM L-glutamine (Gibco BRL), and 100 μM 2-mercaptoethanol (Sigma-Aldrich, St Louis, MO). After removal of LIF, ES cells were cultured on collagen type IV plates (Becton Dickinson, San Jose, CA) at 1×10^4 cells/mL for 2 days. Cells were then disaggregated by trypsin and seeded on OP9 cells at 1×10^5 cells/mL. After 5 days of culture, OP9 cells were removed from ES cells through a Sephadex G10 column (Amersham Bioscience, Uppsala, Sweden), and the ES cells were fractionated by Flk1 and Tie2 expression using FACSvantage (Becton Dickinson). Sorted cells were seeded on OP9 cells at 1000 to 15 000 cells/mL and cultured in the presence of VEGF-C (100 ng/mL; R&D Systems, Minneapolis, MN), VEGF-D (100 ng/mL; R&D Systems), or the caspase inhibitor Z-VAD-fmk (10-100 nM; Calbiochem, La Jolla, CA). When indicated, sorted cells were cultured with recombinant soluble Tie2-Fc fusion protein (30 μg/mL)[19] or recombinant soluble CD4-Fc fusion protein (30 μg/mL).[19]

Immunocytochemistry

Immunocytochemistry was performed essentially as described.[33] Differentiated ES cells cultured on OP9 cells were fixed with 4% paraformaldehyde at 4°C and stained with a rat monoclonal anti-mouse platelet–endothelial cell adhesion molecule 1 (PECAM-1) monoclona antibody (mAb) (MEC13.3; Becton Dickinson) or a rat monoclonal anti-mouse LYVE-1 antibody (ALY7[26]) by the indirect immunoperoxidase method using horseradish peroxidase–conjugated anti-rat IgG. Peroxidase activity was visualized using 3,3′-diaminobenzidine (Dojindo, Kumamoto, Japan). To determine whether Prox-1 was expressed in LYVE-1⁻ cells, we stained cells with biotinylated ALY7 and anti–Prox-1 antibody (Covance, Berkeley, CA). LYVE-1 and Prox-1 expression was detected by reacting with Alexa 488–conjugated Streptavidin (Molecular Probes, Eugene, OR) and Alexa 546–conjugated goat anti-rabbit antibody (Molecular Probes), respectively. For nuclear staining, cells were treated with TOTO3 (Molecular Probes). Stained cells were visualized by fluorescence microscopy. Stained and unstained cells were visualized by fluorescent microscopy (IX71) with either UplanApo 4×/0.13 NA, 10×/0.40 NA, or 20×/0.70 NA objectives (Olympus, Tokyo, Japan). Images were further processed with Adobe

Photoshop (Adobe Systems, San Jose, CA). For DiI labeling, 10 μg/mL DiI-Ac-LDL (Molecular Probes) was added to differentiated ES cells on OP9 cells and adherent cells were incubated for 4 hours at 37°C. After removing the media containing DiI-Ac-LDL, cells were washed and stained with anti–PECAM-1 (FITC-conjugated; Becton Dickinson) or anti–LYVE-1 (biotin-conjugated) plus FITC-conjugated Streptavidin (Becton Dickinson). Uptake of DiI-Ac-LDL by blood vessel endothelial cells (PECAM-1⁺ cells) and lymphatic endothelial cells (LYVE-1⁺ cells) was visualized using a standard rhodamine excitation emission filter (Olympus, Tokyo, Japan).

RT-PCR analysis

Total RNA was extracted from cells using RNeasy Kit (Qiagen, Hilder, Germany). Isolated RNA was reverse-transcribed using an reverse transcriptase (RT) for polymerase chain reaction (PCR) Kit (Clontech, Palo Alto, CA). cDNAs were amplified using Taq polymerase (TaKaRa, Kyoto, Japan). Sequences of gene-specific primers for RT-PCR were as follows: *GATA2*, 5′-(acacaccacccgatacccacctat), 3′-(cctacgccatggcagtcaccatgct); *SCL*, 5′-(cgcggatccacggagcggccgccgagcgcg), 3′-(cggaattccgcgccgcactactt-tggtgtg); c-myb, 5′-(gacagaagaggaggacagaatca), 3′-(tctcagggtcttcgtcgt-tatag); *AML1*, 5′-(ccagcaagctgaggagcggcg), 3′-(cggatttgtaaagacggtga); *Tie2*, 5′-(ttagttctctgtggagtcag), 3′-(aggccctgagttcttcactc); *Flk1*, 5′-(agaacac-caaaagagaggaaacg), 3′-(gcacacaggcagaaaccagtag); *VEGFR3*, 5′-(gctaccact-gctactacaag), 3′-(gataatcccagtcgaaggtg); *Prox1*, 5′-(aagtggttcagcaatttccg), 3′-(tgaccttgtaaatggccttc); *LYVE1*, 5′-(ttcctcgcctctatttggac), 3′-(tctgttgtct-gcgtttcatcc); *Pdpn*, 5′-(gtgccagtgttgttctgggt), 3′-(tctgttgtctgcgtttcatcc); *Evi1*, 5′-(aatatgagtcatgccaaccc), 3′-(cttggtgtactgacatcatc); and *GAPDH*, 5′-(aatcccatcaccatcttcca), 3′-(ccaggggtcttactccttg).

PCR products were separated on a 1.2% agarose gel and gels were stained with ethidium bromide.

Quantitative RT-PCR analysis

Total RNA was isolated from 10^4 cells and cDNA was reverse-transcribed using Superscript III (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The expression levels of Ang1, Ang2, and Ang3 were analyzed by quantitative (Q) RT-PCR using a LightCycler instrument (Roche Diagnostics, Mannheim, Germany) with LightCycler software version 3.5. cDNA was amplified for Q-PCR using SYBR Green I (Sigma-Aldrich) to detect PCR product. cDNA (2 μL) was used in a 20-μL final volume reaction containing 10 μL SYBR Premix Ex Taq (TaKaRa), 0.4 μM *Ang1* forward (5′-GCCTTTGCACTAAAGAAGGTGTTTT-3′), and 0.4 μM *Ang1* reverse (5′-ATACATCCGCACAGTCTCGAAATG-3′). The LightCycler was programmed to run an initial denaturation step at 95°C for 10 seconds followed by 45 cycles of denaturation (95°C for 5 seconds) and extension (60°C for 20 seconds), monitoring the synthesis of product at the end of the extension step of each cycle. The same conditions were used with primers *Ang2* forward (5′-AGGAGATCAAGGCCTACTGT-GACA-3′) and *Ang2* reverse (5′-GCTCCCGAA GCCCTCTTTG-3′), and *Ang3* forward (5′-GTTCCAGGACTGTGCAGAGATCA-3′) and *Ang3* reverse (5′-TCTCCATGTCACAGAACACCTTGAG-3′). The Ang1, Ang2, and Ang3 values were normalized against mouse β-actin (forward 5′-CAGCCTTCCTTCT-TGGGTATGG-3′; reverse 5′-CTGTGTTGGCATAGAGGTC TTTACG-3′).

Flow cytometric analysis and cell sorting

The following mAbs used for flow cytometry were purchased from Becton Dickinson: anti-CD34 (RAM34), anti–c-Kit (ACK45), anti–Sca-1 (E13-161.7), anti–CD45 (30-F11), anti–Flk1 (Avas12α1), anti–PECAM-1 (MEC13.3), anti–Mac-1 (M1/70), anti–Gr-1 (RB6-8C5), and anti–TER-119. Also used were anti-Tie2 mAb (TEK4)[34] and anti–LYVE-1 mAb (ALY7).[26] Fluorescence-activated cell sorting (FACS) analysis was performed on a FACSvantage (Becton Dickinson).

Progenitor assay by methylcellulose culture

Tie2⁺Flk1⁺ or Flk1⁺ cells derived from ES cells were embedded in 1 mL alpha medium containing 1.3% methylcellulose (1500 cp; Sigma-Aldrich), 30% fetal calf serum (FCS), 1% deionized bovine serum albumin (BSA;

Sigma-Aldrich), 0.1 mM 2-mercapto-ethanol (Sigma-Aldrich), 10 ng/mL stem cell factor (SCF; PeproTech EC, London, United Kingdom), 10 ng/mL recombinant mouse interleukin-3 (IL-3; PeproTech EC). 10 ng/mL recombinant human IL-6 (PeproTech EC), and 2 U/mL recombinant human erythropoietin (Epo; Chugai Pharmaceutical, Tokyo, Japan). Cells were cultured in a 35-mm culture dish and incubated at 37°C in a humidified atmosphere with 5% CO_2.

Statistics

Data are expressed as means plus or minus standard deviation (SD). Statistical analysis was conducted using the Student t test. Statistical significance was defined as a P value less than .05.

Results

In vitro differentiation of ES cells

In order to analyze the function of Tie2 in the development of LECs as well as BECs and hematopoietic cells, we developed a cell culture system for ES cell differentiation. After removal of LIF, E14 ES cells were cultured on collagen type IV plates for 2 days to initiate differentiation to a mesoderm lineage; subsequently, cells were transferred to OP9 stromal cells. Markers of both endothelial and hematopoietic cells, Sca-1, c-kit, and CD34, were expressed in undifferentiated ES cells (Figure 1A). Although 1% of ES cells expressed Flk1 on collagen plates, Tie2 was not expressed in Flk1⁺

Figure 1. Mesodermal differentiation of ES cells on OP9 stromal cells. (A) E14 ES cells were cultured on collagen type IV plates for 2 days, and then all cells were cultured on OP9 cells for 9 days. The expression of CD34, c-Kit, Sca-1, CD45, Flk1, and Tie2 in ES cell–derived cells was analyzed at the indicated time points by flow cytometry. Stained $Tie^{-/-}$ cells are represented by purple shaded histograms. Unstained controls are represented by green lines. The percentages of cells in each quadrant are indicated. (B) Gene expression of fractionated cells shown in A (R3-R6) was analyzed by RT-PCR. (C) Fractionated cells (20 000; R3-R6) were cultured on OP9 cells. On day 7 of culture, hematopoietic clusters (red arrowheads) were developed only from the R3 fraction. In other fractions (R4-R6) hematopoietic clusters were not developed, but embryoid body–like colonies (blue arrowheads) developed from R5 and R6 fractions. Cells were analyzed at low (×40) magnification.

cells (data not shown). Following transfer of cells on collagen plates to OP9 cells, 8% of Flk1⁺ cells expressed Tie2 on day 3 of culture. Numbers of Tie2⁺ cells increased until day 5 of culture, when Tie2 expression was maximal; thereafter, both expression levels and numbers of Tie2⁺ cells gradually decreased. At day 9 of culture, cells cocultured with OP9 cells began expressing CD45, a marker of all hematopoietic cells except mature erythrocytes. Since Tie2⁺ cells appeared just before CD45⁻ hematopoietic cells, Tie2⁺ cells in the Flk1⁺ cell fraction may represent hematopoietic progenitor cells. We examined other ES strains, such as TT2 and R1 cells, using the same culture conditions, and confirmed that these strains showed a similar mesodermal phenotypes as E14 cells (data not shown).

Development of hematopoietic, lymphatic endothelial, and blood vessel endothelial cells from Flk1⁺Tie2⁺ cells

To analyze the differentiating potential of ES-derived cells, we fractionated cells on day 5 of culture using Tie2 and Flk1 mAb as shown in Figure 1A (bottom panel, R1-R4). Expression profiling of transcription factors specific for hematopoietic or endothelial cells was undertaken by RT-PCR (Figure 1B). Expression levels of GATA-2, SCL, and AML-1 in the Tie2⁻Flk1⁺ fraction (R3) were 1.7-, 2.5-, and 1.7-fold higher than those in the Tie2⁻Flk1⁻ fraction (R4), respectively. In the Flk1⁻ fraction (R5 and R6), expression levels of these genes were much lower compared with the Tie2⁻Flk1⁺ fraction (R4), suggesting that the Tie2⁺Flk1⁺ fraction may contain committed progenitors of hematopoietic and endothelial lineages. Cells (20 000) fractionated by Flk1 and Tie2 expression were cultured on OP9 cells (Figure 1C), and hematopoietic clusters formed only from the Tie2⁺Flk1⁺ fraction (R3) (Figure 1C, red arrowheads). Hematopoietic clusters were not detected in Flk1⁺Tie2⁻ (R4), Flk1⁻Tie2⁺ (R5), or Flk1⁻Tie2⁻ fractions (R6). The number of hematopoietic progenitors in each fraction was estimated by colony-forming assays in methylcellulose culture (Figure 2A-B). BECs were detected by staining with PECAM-1 mAb (Figure 2C). PECAM-1⁺ endothelial cells were spindle shaped and took up an acetyl-LDL (Figure 2D). To detect LECs, we generated a mAb against LYVE-1 (ALY7), a receptor for extracellular matrix glycosaminoglycan. Using this mAb, we detected LYVE-1⁺ cells on OP9 cells (Figure 2E). These cells also took up acetyl-LDL (Figure 2F), and 65% of them expressed Tie2 (Figure 2I). The lymphatic identity of these cells was further demonstrated by RT-PCR, using primers specific for LEC-enriched genes, namely, Pdpn, VEGFR3, and Prox1 (Figure 2J). Furthermore, LYVE-1⁺ cells coexpressed Prox-1 (Figure 2K). The presence of VEGFR-3 transcripts in these cultures provided the impetus for us to add the lymphatic growth factors, VEGF-C and VEGF-D. The addition of these factors resulted in a marked increase in the number of LYVE-1⁺ cells (Figure 2L) and an increase in the size of monolayers (VEGF-C, Figure 2G; VEGF-D, Figure 2H). These findings suggest that LYVE-1⁺ cells derived from ES cell differentiation cultures express many genes that are restricted to or enriched in LECs.

That Tie2 is required for these cell types is supported by the more than 10-fold increase in hematopoietic, blood vessel endothelial, and lymphatic endothelial colonies from Tie2⁺Flk1⁺ cells compared with Tie2⁻Flk1⁺ or Tie2⁻Flk1⁻ cell fractions (Table 1). To determine which cells produce the Tie2 ligands Ang1, Ang2, and Ang3 in order to support Tie2⁺ cells, we performed quantitative expression analysis for Ang1, Ang2, and Ang3. As shown in Figure 2M, OP9 cells and LYVE-1⁻ ES-derived cells expressed Ang1 but not Ang2 or Ang3. Although low expression levels of
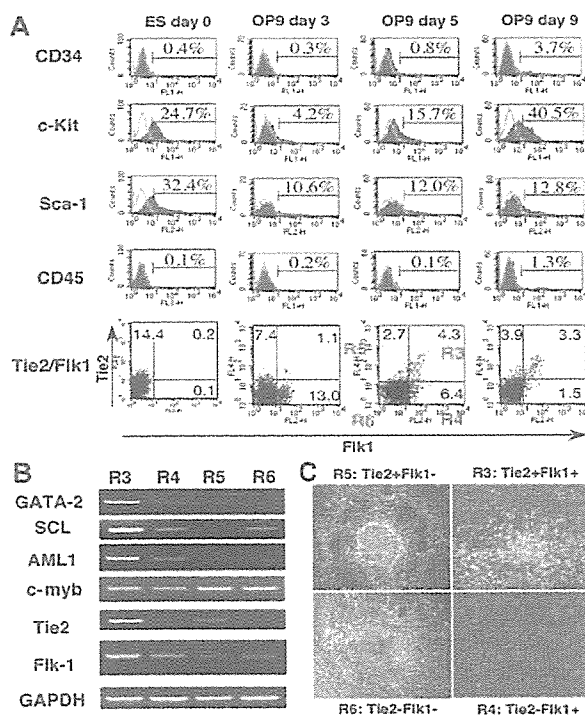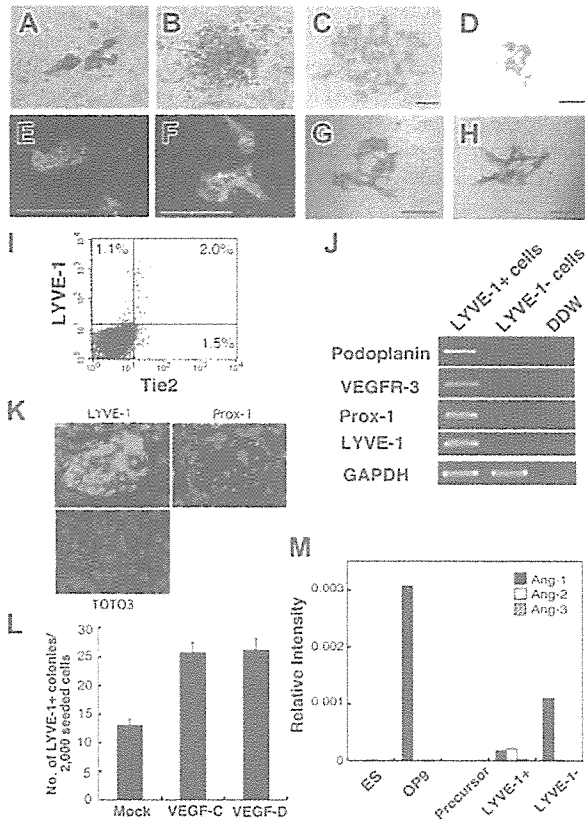
Figure 2. Development of lymphatic endothelial cells from ES cells. The Tie2⁻Flk1⁻ fraction of ES cell–derived cells formed erythroid colonies (A) and granulocyte-macrophage colonies (B) in methylcellulose. Vascular and lymphatic endothelial cells were stained with PECAM-1 mAb (C) and LYVE-1 mAb (D), respectively. PECAM-1⁺ cells (green, panel E) and LYVE-1⁺ cells (green, panel F) took up acetyl-LDL (red, panels E and F). Scale bars, 10 μm. (I) Expression of LYVE-1 and Tie2 in differentiated cells derived from ES cells on OP9 cells on day 6 of culture was analyzed by flow cytometry. The percentages of cells in each quadrant are indicated. (J) Lymphatic-specific genes, Pdpn, VEGFR-3, and Prox-1 in LYVE-1⁺ and LYVE-1⁻ cells were analyzed by RT-PCR. (K) Immunostaining at high magnification (× 200) showed that LYVE-1⁺ cells (green) coexpressed Prox-1 (red). TOTO3 (blue) was used to stain nuclei. (L) In the presence of VEGF-C and VEGF-D (100 ng/mL each), the number of LYVE-1⁺ colonies increased to 2 times that of mock-treated cells. Colonies were also larger in the presence of VEGF-C and VEGF-D (panels G and H, respectively). Results are expressed as the mean ± SD. (M) OP9 and ES cell–derived LYVE-1⁻ cells expressed Ang1. LYVE-1⁺ cells expressed low levels of Ang1 and Ang2. Ang1, Ang2, and Ang3 were not detectable in ES cells and lymphatic precursors (Flk1⁺ cells derived from ES cells).

Ang1 and Ang2 were detected in LYVE-1⁺ cells. ES cells and lymphatic precursor cells (ES cell–derived Flk1⁺ cells) did not express Ang's. These results suggest that Ang1 derived from OP9 cells might affect the growth or survival of Tie2⁺ cells.

Table 1. Frequency of hematopoietic and endothelial progenitors of differentiated ES cells

	Erythroid colonies from 30 000 cells	GM colonies from 30 000 cells	Vascular endothelial colonies from 1500 cells	Lymphatic endothelial colonies from 2000 cells
Tie2⁺Flk1⁺	11.3 ± 6.4	11.7 ± 2.1	47.5 ± 5.3	24.3 ± 2.5
Tie2⁻Flk1⁺	0	0	5.2 ± 1.8	0
Tie2⁺Flk1⁻	0	0	0	0
Tie2⁻Flk1⁻	0	0	0	0
Bulk	0	0	2.7 ± 1.5	1.3 ± 0.6

Development of hematopoietic and endothelial cells from Tie2-deficient ES cells

To clarify the function of Tie2 during hematopoietic and endothelial differentiation from ES cells, the differentiation capacity of Tie2⁻/⁻ ES cells was examined using our culture system. Tie2⁻/⁻ ES cells grew normally on MEF feeder layer cells (data not shown), and differentiated cells grown on OP9 cells were analyzed by flow cytometry. The frequency of cells expressing Flk1 in the Tie2⁻/⁻ ES cells was similar to that seen in Tie2⁺/⁻ cells (Figure 3A). RT-PCR of RNA from this fraction for expression of the genes involved in development of hematopoietic and endothelial cells revealed no remarkable differences between Tie2⁺/⁻ and Tie2⁻/⁻ cells (Figure 3B). To analyze hematopoietic development of Tie2⁻/⁻ ES cells, we calculated the number of hematopoietic clusters formed on OP9 cells at day 7 of culture. The number and size of hematopoietic clusters of Tie2⁻/⁻ ES cells were the same as those of Tie2⁺/⁻ cells (Figure 3C, and data not shown). When the Tie2⁻/⁻ hematopoietic clusters were transferred to fresh OP9 cells for an additional week, normal proliferating hematopoietic cells were detected by flow cytometry. Mature Mac-1–, Gr-1–, and Ter119–positive hematopoietic cells were differentiated from Tie2⁻/⁻ ES cells (Figure 3D), and the frequency of mature hematopoietic cells was similar to that seen in Tie2⁺/⁻ cells (data not shown), suggesting that Tie2 is not essential for development of hematopoietic cells. By contrast, Tie2⁻/⁻ ES cells were severely defective in forming blood vessel and lymphatic endothelial colonies on OP9 cells at day 6 of culture. The number of such blood vessel and lymphatic endothelial colonies was approximately one
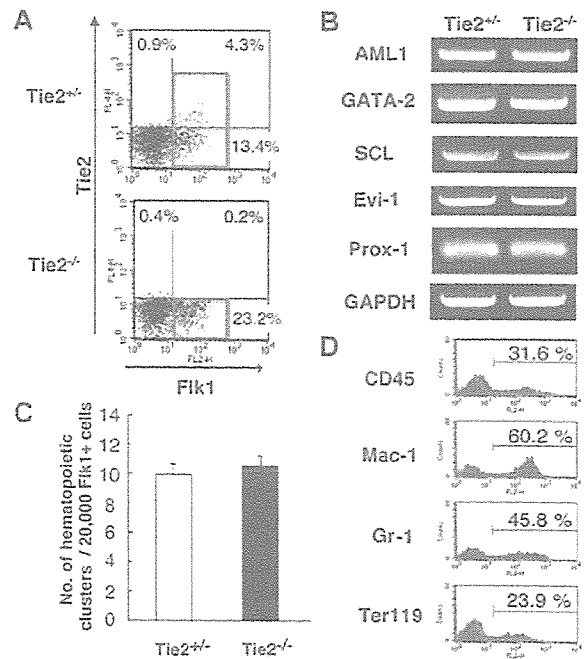

Figure 3. Normal development of hematopoietic cells from Tie2⁻/⁻ ES cells. (A) FACS analysis of the expression of Flk1 and Tie2 in Tie2⁻/⁻ and Tie2⁺/⁻ ES cell–differentiated cells. Red squares show the Flk1⁺ fraction. The percentages of cells in each quadrant are indicated. (B) Expression of genes associated with mesoderm in Flk1⁺ cells shown in panel A (red squares) was analyzed by RT-PCR. (C) Flk1⁺ cells (20 000) were cultured on OP9 cells. The number of hematopoietic clusters from Tie2⁻/⁻ and Tie2⁺/⁻ cells at day 7 of culture was calculated. Results are expressed as the mean ± SD. (D) Tie2⁻/⁻ hematopoietic clusters were cultured for an additional 7 days on fresh OP9 cells. The expression of CD45, Mac-1, Gr-1, and Ter119 was analyzed by flow cytometry. ES cell–derived hematopoietic cells are represented by purple shaded histograms; unstained controls, by green lines.
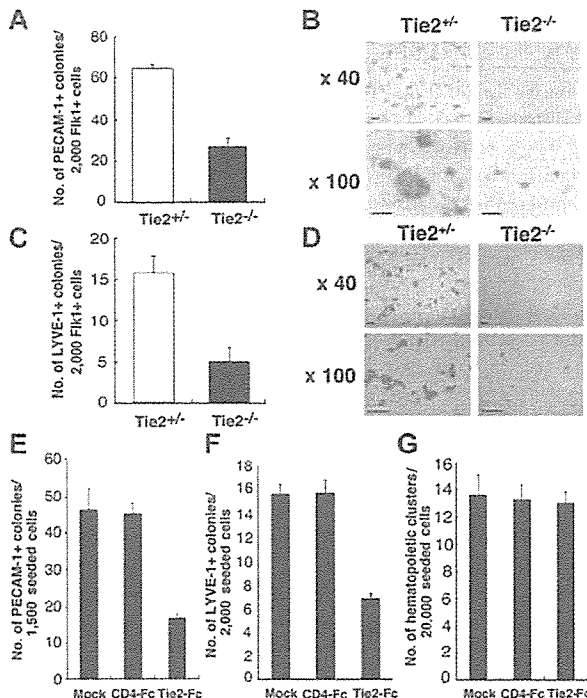
Figure 4. Lymphatic and blood vessel endothelial cell development from Tie2$^{-/-}$ ES cells. (A) Flk1 cells (2000) were cultured on OP9 cells. At day 7 of culture, the number of vascular endothelial colonies developed from Tie2$^{+/-}$ (□) and Tie2$^{-/-}$ (■) ES cells was calculated. (B) Vascular endothelial colonies were stained with PECAM-1 mAb and analyzed at low (× 40) and high (× 100) magnification. (C) The number of lymphatic endothelial colonies developed from Tie2$^{-/-}$ (□) and Tie2$^{-/-}$ (■) ES cells was calculated. (D) Lymphatic endothelial colonies were stained with LYVE-1 mAb and analyzed at low (× 40) and high (× 100) magnification. Scale bars in panels B and D, 20 μm. In the presence of Tie2-Fc (30 μg/mL), the number of vascular and lymphatic endothelial colonies was decreased (panels E and F, respectively). (G) Hematopoietic cluster formation was not affected by exogenous soluble Tie2-Fc (30 μg/mL). Exogenous soluble CD4-Fc (30 μg/mL) served as the control. Results in panels A, C, and E-G are expressed as the mean ± SD.

third that observed from Tie2$^{+/-}$ cells (Figure 4A-B), and colony size was smaller than that seen from Tie2$^{+/-}$ cells (Figure 4C-D). These results suggest that Tie2 function is required for development of BECs and LECs, but not of hematopoietic cells. To confirm these findings, we added soluble Tie2-Fc fusion protein to the culture media of wild-type ES cells to block Tie2 signaling. As shown in Figure 4E-G, the number of vascular and lymphatic endothelial cell colonies derived from wild-type ES cells in the presence of soluble Tie2-Fc fusion protein decreased to one half to one third of the mock-treated cells, while the number of hematopoietic clusters was not affected by soluble Tie2-Fc fusion protein. We did not detect such inhibition in the presence of soluble CD4-Fc fusion protein (Figure 4 E-F).

Tie2 signaling is crucial for antiapoptotic signaling in the development of lymphatic and blood vessel endothelial cells

To further analyze the mechanisms underlying defective proliferation of BECs and LECs from Tie2$^{-/-}$ ES cells, we analyzed expression of blood vessel– and lymphatic-specific markers PECAM-1 and LYVE-1, respectively, by flow cytometry at days 2, 4, and 6 of culture (Figure 5A). Cells expressing PECAM-1 and LYVE-1 in Tie2$^{-/-}$ ES cells decreased over 6 days. PECAM-1$^+$ cells in Tie2$^{-/-}$ cells were approximately one third the number of those in Tie2$^{+/-}$ cells at day 6 of culture, as was the case with LYVE-1 cells (Figure 5B-C). This finding suggested that Tie2$^{-/-}$-mediated signaling

protects endothelial cells from cell death. To test this possibility, we treated Tie2$^{-/-}$ cells with the caspase inhibitor Z-VAD-fmk. As shown in Figure 5B and C, lymphatic and blood vessel endothelial colony formation was rescued in the presence of Z-VAD-fmk in a dose-dependent fashion. In the presence of 50 nM of Z-VAD-fmk, the number of lymphatic endothelial colonies from Tie2$^{-/-}$ cells was rescued to 60% of that from Tie2$^{+/-}$ cells (Figure 5C), although the size of Tie2$^{-/-}$ lymphatic endothelial colonies remained small (Figure 5D). The formation of blood vessel endothelial colonies was similar in the presence of Z-VAD-fmk (50 nM; Figure 5C). These findings suggest that Tie2 signaling is crucial for LEC and BEC development and mediates antiapoptotic signaling during ES cell differentiation.

Discussion

Although we have demonstrated that Tie2 is expressed in the vitelline artery,[19] the AGM region,[4] and fetal liver,[20] the function of Tie2 in development has not been elucidated. The early death of Tie2$^{-/-}$ embryos precludes detailed analysis of the role of Tie2 in
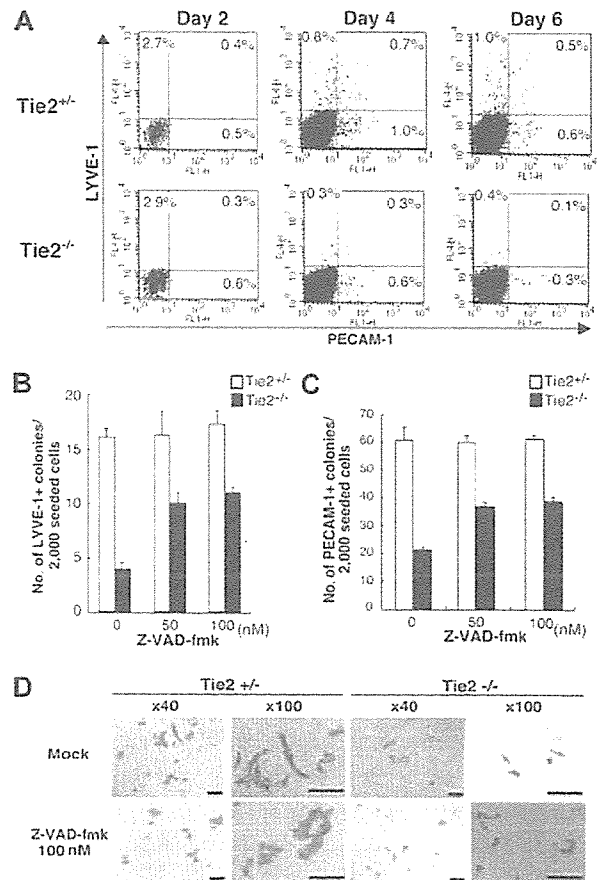

Figure 5. Tie2 signaling in blood vessels and lymphatic endothelial cells protected from apoptosis. (A) Expression of PECAM-1 and LYVE-1 in ES cell–derived cells was analyzed by flow cytometry at days 2, 4, and 6 of culture. The percentages of cells in each quadrant are indicated. (B) Addition of the caspase inhibitor Z-VAD-fmk (50 nM) to the culture media rescued the number of LYVE-1 colonies from Tie2$^{-/-}$ ES cells (■). (C) The number of PECAM-1$^+$ colonies from Tie2$^{-/-}$ ES cells (■) was also rescued in the presence of Z-VAD-fmk (50 nM). Results are expressed as mean ± SD. (D) In the presence of Z-VAD-fmk, the size of Tie2$^{-/-}$ lymphatic endothelial cells was unchanged, although the number of lymphatic colonies was partially rescued. Scale bars, 20 μm.

development of hematopoietic and endothelial lineages. Using an ES cell differentiation system, we have analyzed the development of hematopoietic cells as well as BECs and LECs from $Tie2^{-/-}$ ES cells. At day 8 of culture on OP9 cells, the normal formation of hematopoietic clusters from $Tie2^{-/-}$ cells suggests that Tie2 signaling does not contribute to development of hematopoietic cells from precursors. Furthermore, differentiation of myeloid cells from $Tie2^{-/-}$ hematopoietic clusters was normal. These results indicate that Tie2 is not essential for development of hematopoietic cells from ES cells. Recently, Puri and Bernstein[21] used combined mosaic analysis to demonstrate that Tie receptors are not required for differentiation and proliferation of definitive hematopoietic lineages in the embryo and fetus. Their findings are consistent with what we show in this study. Although our in vitro differentiation experiments suggest that Tie2 is not essential for hematopoiesis during development, Ang1/Tie2 signaling in hematopoietic cells has been reported to function to maintain hematopoietic stem cells in the bone marrow niche where Ang1 is expressed by osteoblasts.[18] Thus Tie2 function in hematopoiesis would seem to be specific for adult bone marrow.

Although much is known about normal and pathologic development of the vascular system,[35] the lack of specific markers has made it difficult to follow the development of the lymphatic system. In order to identify LECs, we recently generated a LEC-specific mAb against LYVE-1.[26] This mAb allowed us to detect LECs in mouse embryos at midgestation and purify these cells. ES cell–derived LYVE-1$^+$ cells express LEC-specific genes, such as $Prox1$, $Pdpn$, and $VEGFR3$, and exhibit DiI-Ac-LDL uptake. Furthermore, VEGF-C and VEGF-D, lymphatic growth factors, increased the number of LYVE-1–positive endothelial colonies from ES cells. These findings indicate that LYVE-1$^+$ cells isolated from these cultures have many characteristics of LECs. Lymphatic vasculature of the thoracic and abdominal viscera have been proposed to arise by endothelial spreading from lymph sacs.[6,36] This proposal is supported by the finding that budding of endothelial cells from the veins of Prox-1 mutant embryos is arrested.[9,10] To clarify the mechanisms of lymphangiogenesis in vitro, we analyzed the function of Tie2. In this study LECs were differentiated from ES cell–derived Tie2$^+$Flk1$^-$ cells, and approximately 1% of Tie2$^+$Flk1$^+$ cells formed lymphatic endothelial colonies on OP9 cells. Our in vitro differentiation assay allows us to clarify the mechanisms of LEC development from its precursor.

Thus far, Tie2 signaling has been reported to be required for proper development and function of the vascular system. Mice lacking Ang2 exhibit lymphatic vessel defects, strongly suggesting a role of Tie2 signaling in lymphangiogenesis.[37] In this study we have shown that LECs and BECs can be differentiated from Tie2$^+$Flk1$^+$ cells, and that 65% of LYVE-1$^+$ LECs express Tie2. To analyze the function of Tie2 in the development of lymphatic

endothelial cells, we performed FACS analysis and immunocytochemistry during differentiation of $Tie2^{-/-}$ ES cells. FACS analysis revealed that $Tie2^{-/-}$ cells expressing LYVE-1 decreased over time as did PECAM-1$^+$ BECs. Treatment with a caspase inhibitor, which specifically inhibits apoptosis, partially rescued defective formation of lymphatic and blood vessel endothelial colonies from $Tie2^{-/-}$ cells, suggesting that both endothelial cells undergo apoptosis in the absence of Tie2 signaling. Although Tie2 signaling contributed to the survival of both LECs and BECs on OP9 cells, treatment with the caspase inhibitor did not affect the size of $Tie2^{-/-}$ colonies. Based on these findings, we propose that Tie2 cooperates with other signaling pathways involved in growth. Although we do not identify these pathways, OP9 cells are known to secrete several growth factors, including VEGF-C and VEGF-D.[38] FACS analysis also revealed that at day 2 of culture, the frequency of LYVE-1$^+$ and PECAM-1$^+$ cells derived from $Tie2^{-/-}$ cells was comparable with frequencies seen in $Tie2^{+/-}$ cells, suggesting that lymphatic and blood vessel endothelial precursors develop normally from $Tie2^{-/-}$ cells. A gene expression study clearly showed that expression levels of Prox-1 in lymphatic precursors (the Flk1$^+$ cell fraction) of $Tie2^{-/-}$ cells were comparable with those seen in $Tie2^{+/-}$ cells, suggesting that Tie2 deficiency does not affect Prox-1 expression. These findings suggest that Tie2 is not essential for development of lymphatic and blood vessel endothelial precursors, although both types of endothelial cells from $Tie2^{-/-}$ cells undergo apoptosis due to lack of Tie2 signaling.

Regarding vascular endothelial cells, in vivo study has shown that mice lacking Ang1 and Tie2 develop a fairly normal primary vasculature, but that this vasculature fails to undergo further normal remodeling.[28,39,40] We have demonstrated that lymphatic endothelial cells express Tie2 in both embryonic and adult settings, and that the activation of Tie2 signaling by Ang1 stimulates both in vivo lymphatic angiogenesis in mouse cornea and in vitro colony formation of lymphatic endothelial cells.[26] Data from both in vitro ES cell differentiation and in vivo embryonic development suggest that Ang/Tie2 signaling may contribute to regulation of lymphatic vessel formation in the development of lymphatic vessels.

In summary, our results derived from induction studies of ES cells have revealed that Tie2 is expressed in the precursors of mesodermal lineages, hematopoietic cells, and endothelial cells. We have also shown that Tie2 is not essential for development of hematopoietic cells, but that it plays an important role in antiapoptotic signaling in lymphatic and blood vessel development.

Acknowledgment

References

1. Sabin F. Studies on the origin of blood vessels and red corpuscles as seen in the living blastoderm of the chick during the second day of incubation. Contribut Embryol. 1920;9:213-262.

2. Kubo H, Alitalo K. The bloody fate of endothelial stem cells. Genes Dev. 2003;17:322-329.

3. Haar JL, Ackerman GA. A phase and electron microscopic study of vasculogenesis and erythropoiesis in the yolk sac of the mouse. Anat Rec. 1971;170:199-223.

4. Hamaguchi I, Huang XL, Takakura N, et al In vitro hematopoietic and endothelial cell development from cells expressing TEK receptor in murine

aorta-gonad-mesonephros region. Blood. 1999;93:1549-1556.

5. Choi K, Kennedy M, Kazarov A, Papadimitriou JC, Keller G. A common precursor for hematopoietic and endothelial cells. Development. 1998;125:725-732.

6. Sabin FR. On the origin of the lymphatic system from the veins, and the development of the lymph hearts and thoracic duct in the pig. Am J Anat. 1902;1:367-389.

7. Oliver G, Detmar M. The rediscovery of the lymphatic system: old and new insights into the development and biological function of the lymphatic vasculature. Genes Dev. 2002;16:773-783.

8. Jeltsch M, Kaipainen A, Joukov V, et al. Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. Science. 1997;276:1423-1425.

9. Wigle JT, Oliver G. Prox1 function is required for the development of the murine lymphatic system. Cell. 1999;98:769-778.

10. Wigle JT, Harvey N, Detmar M, et al. An essential role for Prox1 in the induction of the lymphatic endothelial cell phenotype. EMBO J. 2002;21:1505-1513.

11. Shalaby F, Rossant J, Yamaguchi TP, et al. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature. 1995;376:62-66.

12. Shalaby F, Ho J, Stanford WL, et al. A requirement for Flk1 in primitive and definitive hematopoiesis and vasculogenesis. Cell. 1997;89:981-990.

13. Millauer B, Wizigmann-Voos S, Schnurch H, et al. High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. Cell. 1993;72:835-846.

14. Dumont DJ, Fong GH, Puri MC, Gradwohl G, Alitalo K, Breitman ML. Vascularization of the mouse embryo: a study of flk-1, tek, tie, and vascular endothelial growth factor expression during development. Dev Dyn. 1995;203:80-92.

15. de Vries C, Escobedo JA, Ueno H, Houck K, Ferrara N, Williams LT. The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. Science. 1992;255:989-991.

16. Dumont DJ, Gradwohl GJ, Fong GH, Auerbach R, Breitman ML. The endothelial-specific receptor tyrosine kinase, tek, is a member of a new subfamily of receptors. Oncogene. 1993;8:1293-1301.

17. Iwama A, Hamaguchi I, Hashiyama M, Murayama Y, Yasunaga K, Suda T. Molecular cloning and characterization of mouse TIE and TEK receptor tyrosine kinase genes and their expression in hematopoietic stem cells. Biochem Biophys Res Commun. 1993;195:301-309.

18. Arai F, Hirao A, Ohmura M, et al. Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. Cell. 2004;118:149-161.

19. Takakura N, Huang XL, Naruse T, et al. Critical role of the TIE2 endothelial cell receptor in the development of definitive hematopoiesis. Immunity. 1998;9:677-686.

20. Hsu HC, Ema H, Osawa M, Nakamura Y, Suda T, Nakauchi H. Hematopoietic stem cells express Tie-2 receptor in the murine fetal liver. Blood. 2000;96:3757-3762.

21. Puri MC, Bernstein A. Requirement for the TIE family of receptor tyrosine kinases in adult but not fetal hematopoiesis. Proc Natl Acad Sci U S A. 2003;100:12753-12758.

22. Koblizek TI, Weiss C, Yancopoulos GD, Deutsch U, Risau W. Angiopoietin-1 induces sprouting angiogenesis in vitro. Curr Biol. 1998;8:529-532.

23. Papapetropoulos A, Garcia-Cardena G, Dengler TJ, Maisonpierre PC, Yancopoulos GD, Sessa WC. Direct actions of angiopoietin-1 on human endothelium: evidence for network stabilization, cell survival, and interaction with other angiogenic growth factors. Lab Invest. 1999;79:213-223.

24. Kwak HJ, So JN, Lee SJ, Kim I, Koh GY. Angiopoietin-1 is an apoptosis survival factor for endothelial cells. FEBS Lett. 1999;448:249-253.

25. Jones N, Voskas D, Master Z, Sarao R, Jones J, Dumont DJ. Rescue of the early vascular defects in Tek/Tie2 null mice reveals an essential survival function. EMBO Rep. 2001;2:438-445.

26. Morisada T, Oike Y, Yamada Y, et al. Angiopoietin-1 promotes LYVE-1-positive lymphatic vessel formation. Blood. 2005;105:4649-4656.

27. Tammela T, Saaristo A, Lohela M, et al. Angiopoietin-1 promotes lymphatic sprouting and hyperplasia. Blood. 2005;105:4624-4648.

28. Dumont DJ, Gradwohl G, Fong GH, et al Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, tek, reveal a critical role in vasculogenesis of the embryo. Genes Dev. 1994;8:1897-1909.

29. Puri MC, Rossant J, Alitalo K, Bernstein A, Partanen J. The receptor tyrosine kinase TIE is required for integrity and survival of vascular endothelial cells. EMBO J. 1995;14:5884-5891.

30. Yagi T, Tokunaga T, Furuta Y, et al. A novel ES cell line, TT2, with high germline-differentiating potency. Anal Biochem. 1993;214:70-76.

31. Hooper M, Hardy K, Handyside A, Hunter S, Monk M. HPRT-deficient (Lesch-Nyhan) mouse embryos derived from germline colonization by cultured cells. Nature. 1987;326:292-295.

32. Nagy A, Rossant J, Nagy R, Abramow-Newerly W, Roder JC. Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. Proc Natl Acad Sci U S A. 1993;90:8424-8428.

33. Takakura N, Yoshida H, Ogura Y, Kataoka H, Nishikawa S. PDGFR alpha expression during mouse embryogenesis: immunolocalization analyzed by whole-mount immunohistostaining using the monoclonal anti-mouse PDGFR alpha antibody APA5. J Histochem Cytochem. 1997;45:883-893.

34. Yano M, Iwama A, Nishio H, Suda J, Takada G, Suda T. Expression and function of murine receptor tyrosine kinases, TIE and TEK, in hematopoietic stem cells. Blood. 1997;89:4317-4326.

35. Gale NW, Yancopoulos GD. Growth factors acting via endothelial cell-specific receptor tyrosine kinases: VEGFs, angiopoietins, and ephrins in vascular development. Genes Dev. 1999;13:1055-1066.

36. Gray H. In Anatomy of the human body. In: Clemente CD, ed. The Lymphatic System. Philadelphia, PA: Lea and Febiger; 1985:866-932.

37. Gale NW, Thurston G, Hackett SF, et al. Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by Angiopoietin-1. Dev Cell. 2002;3:411-423.

38. Matsumura K, Hirashima M, Ogawa M, et al. Modulation of VEGFR-2-mediated endothelial-cell activity by VEGF-C/VEGFR-3. Blood. 2003;101:1367-1374.

39. Suri C, Jones PF, Patan S, et al. Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. Cell. 1996;87:1171-1180.

40. Sato TN, Tozawa Y, Deutsch U, et al. Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. Nature. 1995;376:70-74.

How to comply with cGMP during early phase of translational cell therapy at academia

Taira Maekawa, Dept of Transfusion Medicine and Cell Therapy, Center for Cell & Molecular Therapy, Kyoto University Hospital

Professor Taira Maekawa, M.D., D.M.Sci., Dept of Transfusion Medicine and Cell Therapy, & Director, Center for Cell & Molecular Therapy, Kyoto University Hospital

1. Cell Processing Center - Concept

This caricature roughly represents the concept of a cell processing center or CPC (Fig. 1). The CPC is mandatory for cell therapy as shown here, in which donors or patients provide their own cells to a reception area. Those cells are then divided or separated into different components and then we introduce a gene or expand them with cytokines by sequencing in order to develop it. Then the quality control unit confirms the safety and sterility of new product and after that we hand it over to the clinical ward. This is the concept of CPC.

2. KUH - Center for Cell & Molecular Therapy

In order to develop the CPC in Kyoto University, we kept a 200-square meter wide space (Fig. 2). My previous office and secretary's room were located in this building. To keep adequate space, I removed my office and secretary's room and some laboratory room to make way for CPC, called the Center for Cell & Molecular Therapy (CCMT).

The CCMT is located on the third floor of the Clinical Building of Kyoto University Hospital, just next door to the Department of Transfusion Medicine.

3. Layout/Floor plan of CCMT

I will now give you a virtual tour of the inside of CCMT (Fig. 3). The entrance to the Center is accessible only to authorized personnel. This is the entrance with security card system (Fig. 4). This is the monitoring room where we can monitor everything. We can monitor the room pressure and temperature and CO_2 levels of incubators and everything else in each room (Fig. 5). We can also monitor the cleanliness of the air in each room. We've installed particle counter in every room so we can monitor easily for any type of air contamination. The air direction is one-way pass through. So this is the room where we prepare pancreatic islet cells. This is the incubator, safety cabinet, and this is COBE 2991 machine. This is the cryo-storage room (Fig. 6). Unlike in your laboratory in France, we have only two liquid-nitrogen tanks in our lab because of limited space. We store samples using two-directional bar-cord system.

4. Products developed in CCMT

We now have about 5 or 6 products (Fig. 7). Two of them have already reached the clinic. One is islet pancreatic transplantation. The other one is dendritic cells for immunotherapy to leukemia

patients. And then we have cardiovascular regeneration, bone tissue regeneration for bone necrosis disease and culture of inner skin. We have already finished the SOP (Standard Operating Procedures) of these two and we are now waiting of the acceptance by the IRB (Institutional Review Board) in our university hospital. Each projects are going to proceed by the responsible doctors of each clinical department, and medical doctors shown in this slides of my department usually consult with these doctors from the corresponding department.

5. News coverage on success of pancreatic islet cell transplantation

Last year we succeeded in performing pancreatic islet cell allo-transplantation from a living donor (Fig. 8). This procedure was done by Dr. Matsumoto as chief of the pancreatic islet cell transplantation team. An article about this success appeared in the online edition of Lancet on April 19 last year. On the same day, Nature Journal cited this study in one of its articles. And then BBC, USA Today, Forbes and other major print and broadcast media around the world made mention of this successful study. Finally, Aljazeera Broadcast of Iraq reported this therapeutic strategy.

6. CGMP elements

Current GMP or cGMP consists of ten elements (Table 1), namely, facility and equipment, production and process controls, personnel management, recordkeeping, calibration, validation, error management, SOPs, labeling and quality control and auditing. So in order to satisfy these ten elements of cGMP, we have to do a lot of very tough work. However, conventional cGMP rules can be preferentially applied for drugs or tablets produced in big pharmaceutical companies.

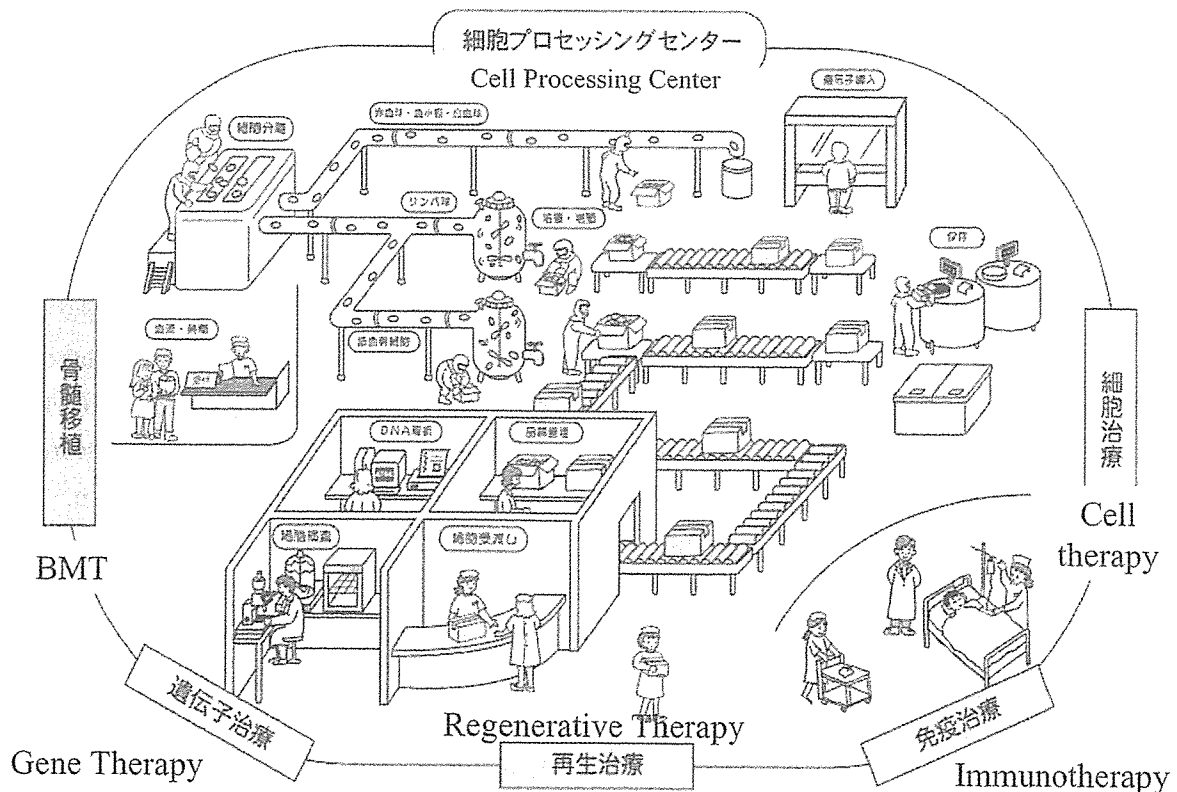Fig. 1　CPC is mandatory for Cell Therapy

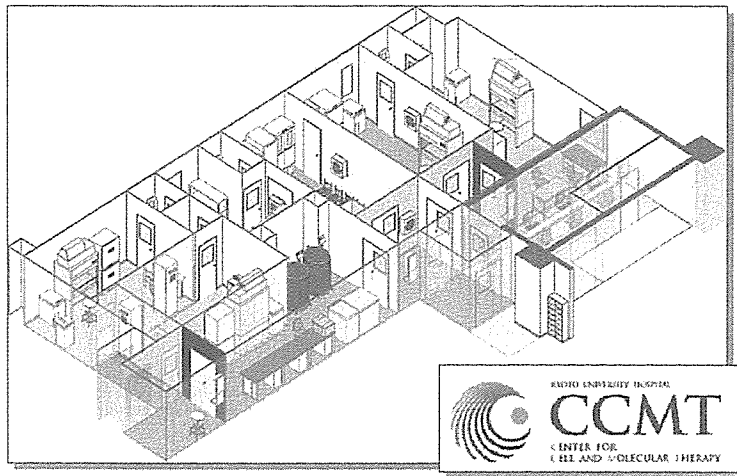Fig. 2 Center for Cell & Molecular Therapy
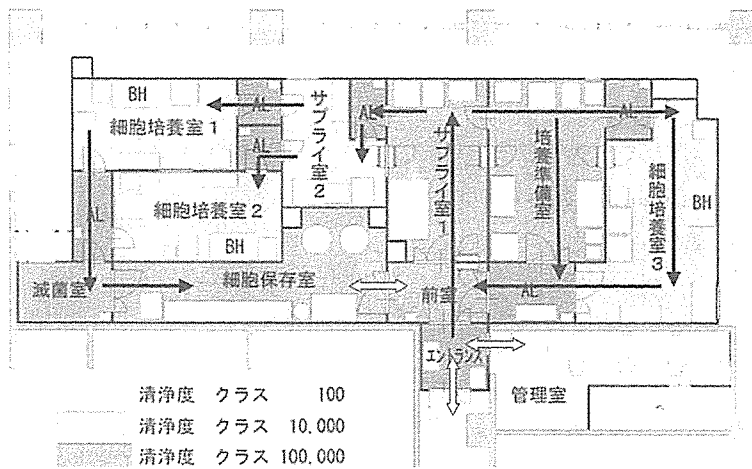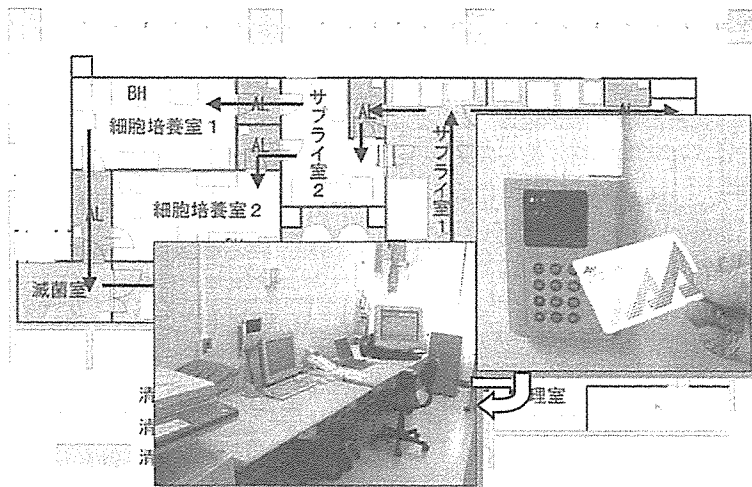

Fig. 3 Inside of CCMT

Fig. 4 Inside of CCMT (continue)

Fig. 5　Inside of CCMT (continue)
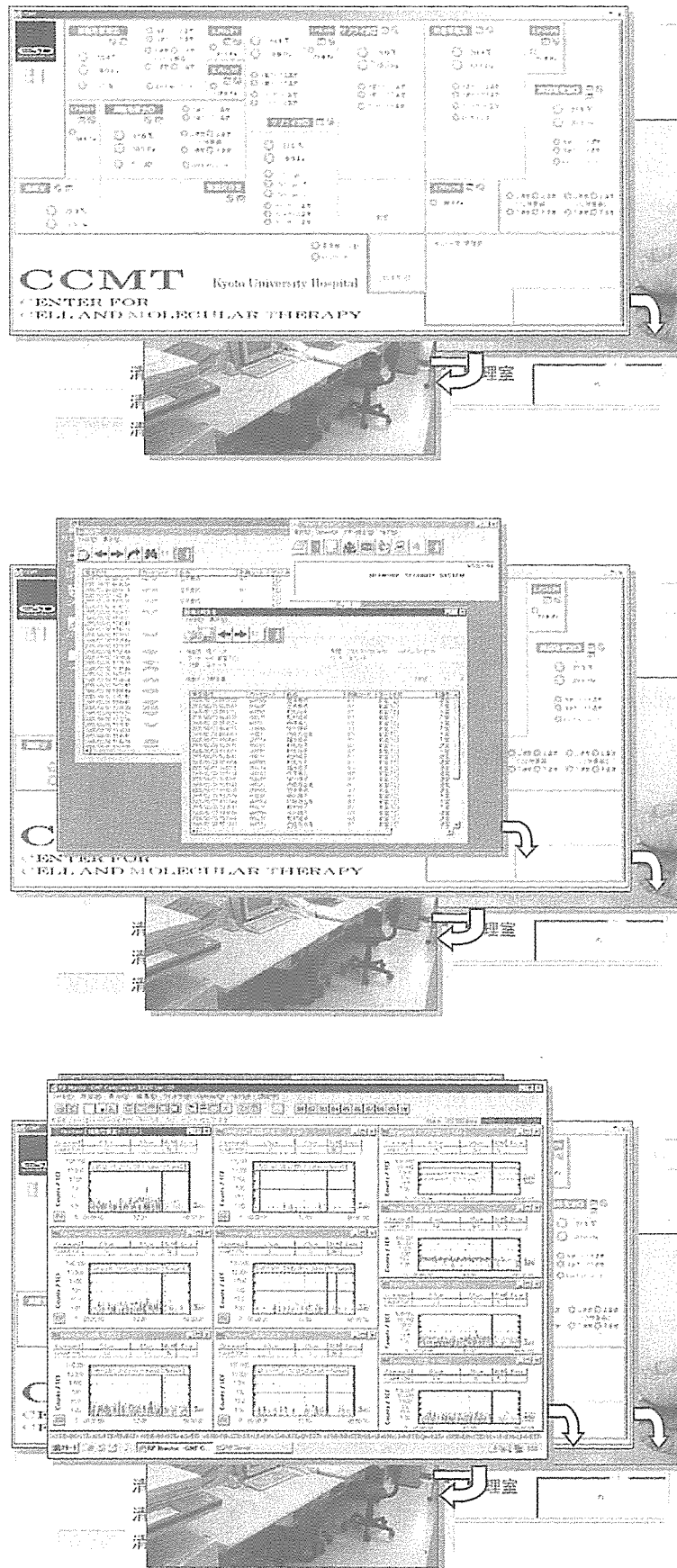
Fig. 6 Inside of CCMT (continue)
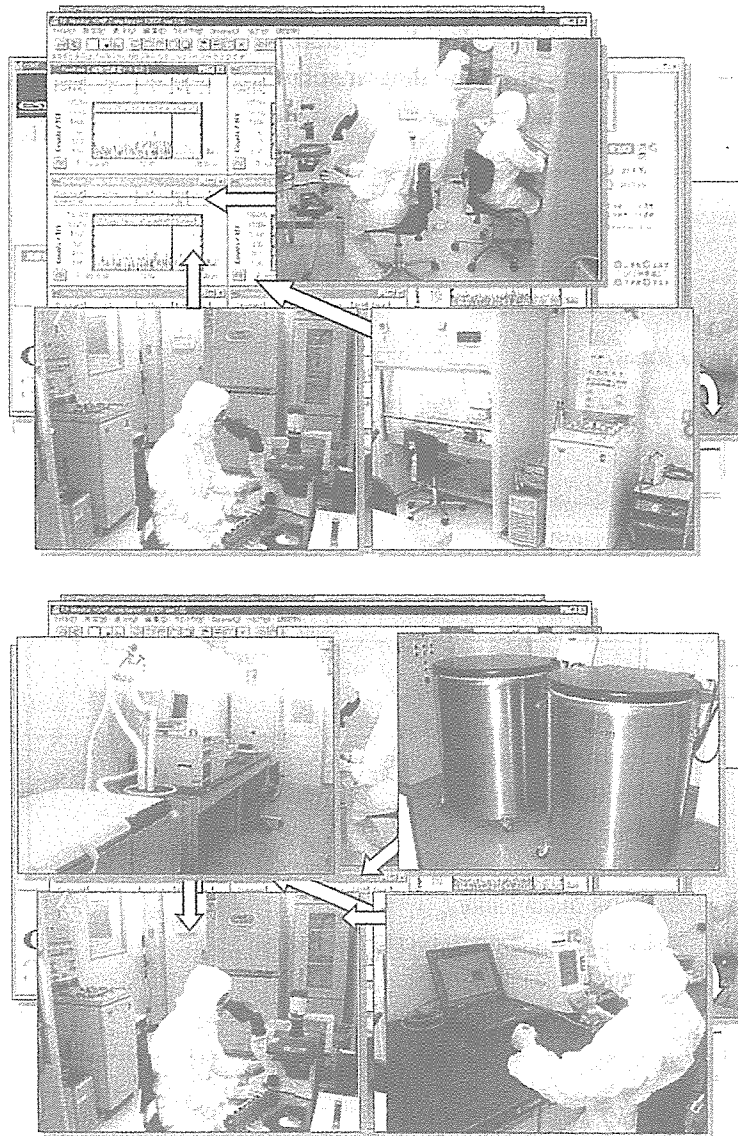
Fig. 7 Cell Therapy Projects at Kyoto University Hospital

Fig. 8 Success of pancreatic Islet Cell Transplantation from Related Living Donar

Table 1 Ten Elements of cGMP

1. Facility and equipment
2. Production and process controls
3. Personnel management
4. Record-keeping
5. Calibration
6. Validation
7. Error management
8. Standard operating procedures (SOPs)
9. Labeling
10. Quality control and auditing

Apply for...

drugs or tablets produced in pharm.

7. Cell processing - special characteristic

I would like to explain the special characteristic of cell processing (Table 2). Cell processing is not the production of conventional pharmaceutical drugs like tablets or injections. And, unlike a pharmaceutical company, the academic institution has not adequate size of staffs for cell processing in general.

8. Special validations in cell processing

We have some impossible validations for cell processing if we apply cGMP regulations used for conventional pharmaceutical drugs (Table 3). For

example, performance qualification (PQ) - PQ for worst case validation using pancreas tissue from donor is impossible, although dry-run or water-run is possible. Another example is process validation. Process validation is required to confirm the quality of products using at least three batches of the product. However, in most cases, cell processing products do not comprise the batch. And there are more.

9. Characteristics of early phase TR cell therapy

In these kinds of research in the academia (Table 4), only very small number of patients is

enrolled in most cases - 5 or 10 patients. We have to support multiple clinical trials - pancreatic islet cells, dendritic cells and so on. But we have to efficiently manage our projects with limited or inadequate size of staffs.

10. Stepwise approach in cGMP development in cell therapy

Rather than cGMP, I propose to adopt the institutional GMP or iGMP in cell therapy. Stepwise approach (Fig. 9) is necessary for development of novel gene and cell therapy of early phase I or translational stage in the academia as regulatory requirements increase with product development. And therefore I think that iGMP is specifically required of cell processing for early phase I study or translational research and should be appropriate for academia.

We tried to develop this iGMP but authorities are concerned that there will be double standard of GMP and they do not like to complete the documentation for this iGMP. But I believe that iGMP should be established to advance translational or exploratory cell therapy in academia. So under this concept, we will develop the cell products in our facility. In future, when these novel therapies could be truly confirmed to be effective after Phase 3 and thereafter patients would be routinely received these treatments, most of these cell products will be produced in a blood center and in

Table 2 Cell Processing is Unique by its "Boutique" Quality

- Cell processing is
    ≠ Production of conventional pharmaceutical drugs

- Academic institution is
    ≠ Pharmaceutical company

Table 3 Impossible Validations for Cell Processing

Performance Qualification (PQ)
    e.g. PQ for worst case validation using pancreas tissue from donor is impossible, although dry-run or water-run is possible

Process Validation (PV)
    e.g. PV requires to confirm the quality of products using at least three batches of them. However, Cell Processing Products do not consist the batch in most cases.

....and more

Table 4 Characteristics of Early Phase (TR) Cell Therapy in Academia

- Enroll very small numbers of patients
- Support multiple clinical trials
- Manage projects with inadequate size staff

some companies and then distributed to the general hospitals like blood products.

11. FDA guidance

　Very recently FDA launched a couple of guidance for industry for early phase clinical trials (Fig. 10). One is entitled INDs-Approaches to Complying with cGMP during Phase 1. This guidance is specific for early phase clinical trials. Another one is Guidance for Industry, Investigators and Reviewers - Exploratory IND studies. But the former document is still in draft form and it is currently open for public comment.

12. FDA guidance for special production situations

　This new guidance (Fig. 11) for industry includes approaches similar to iGMP for Phase 1. There is one section for special production situations which covers multi-product facilities, biological and biotechnological products such as gene therapy or cellular therapy products. So in this guidance, they have included regulations that are more appropriate for cell therapy studies. For example, they say that "we understand that products with a short shelf-life may have to be released

Fig. 9　Stepwise approach: regulatory requirements increase with product development
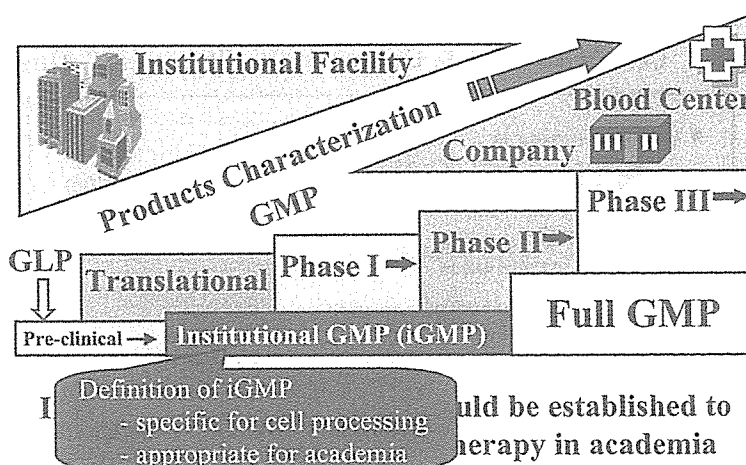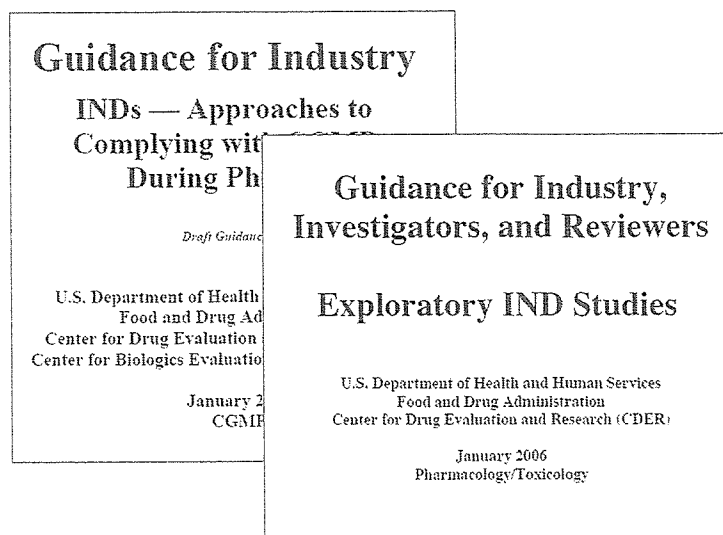

Fig. 10　Recent Documents from FDA

while results of the sterility test are pending based on results from other relevant tests." So I think that this is similar to the concept of iGMP or stepwise approach using cGMP for translational studies.

13. Situation of drug development in Japan

In Japan, it is usually the pharmaceutical companies who decide the priority of projects and only initiate the preferential project worth while developing for the clinical settings, that means it promise to have a large market value (Fig. 12). However, the academia has a number of seed compounds that can be developed into therapeutic products with interests by researchers or physician scientists. Even if we could successfully proceed the appropriate project as a translational research, we could not proceed to the next stage such as phase 2 or phase 3 on the basis of fruitful results of translational research unless we would apply this clinical trials beforehand to the Pharmaceuticals and Medical Devices Agency (PMDA) for review of the protocol. When we would not apply to the PMDA for review of the protocol beforehand, we have to return to the beginning of preclinical or phase 1 studies even though we obtained the good results in the translational re-

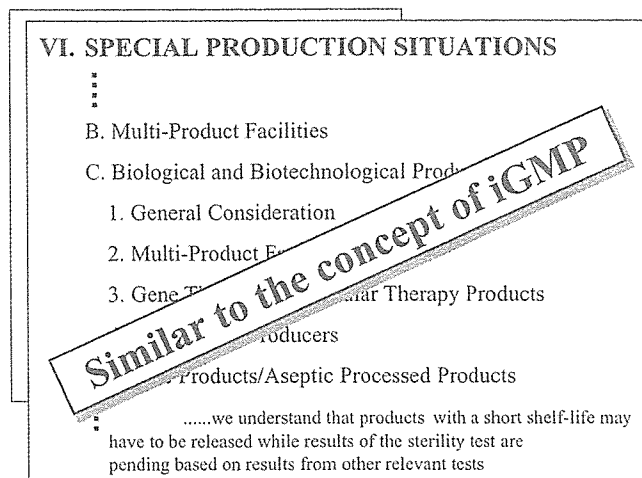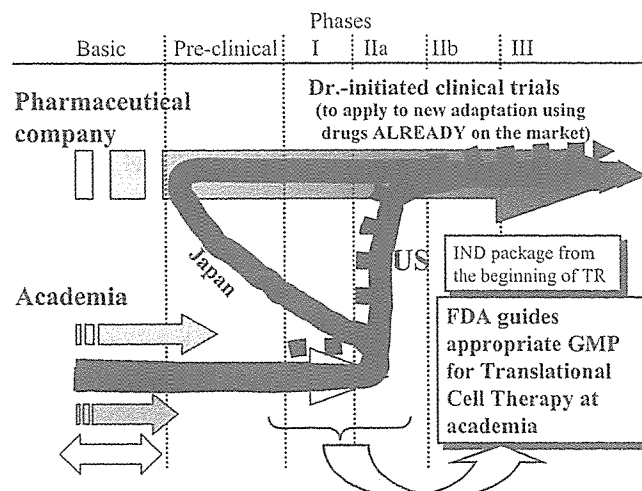Fig. 11 VI. Special production situations

Fig. 12

search and we intended to develop this new therapeutic procedures to conventional treatment. The reason of this return may be lack of reliability, safety, and ethical aspects of the protocol even if we apply this to the IRB (institutional review board), because most of university IRB has no ability to review the protocol according to the guideline of ICH-GCP and cGMP

However, in the United States, this kind of project can go to Phase 2A clinical development because the FDA has GMP guidance for translational cell therapy research appropriate for academia. They require IND package at the beginning of the translational research, and they review the protocol beforehand to check its reliability, safety, and ethical aspects of the project according to the guideline of ICH-GCP and cGMP.

In Japan, if we do this kind of project without application for and reviewed by the PMDA, it will be considered to be a non-registered clinical trial. The PMDA and the Ministry of Health, Labour and Welfare do not accept such non-registered clinical trials and do not allow us to proceed for further development. There are one exception called "in house product". When we produce cellular products in the university hospital and administer them to the patients of the university hospital under our responsibility, there are no regulations governed by the PMDA or authority. In such situations, the quality of the "in house" cellular products should be solely assured by and depend on the investigators of the university. If we take this track, we cannot bring this therapeutic strategies to the public clinical settings covered by health insuarance in Japan.

Recently, we were able to conduct investigator-initiated clinical trials Under the present laws and regulations in Japan, I think we had better to intelligently and flexibly use this track for the development of novel cellular therapy. And in order to do so, we must prepare cellular products under cGMP guideline considering the characteristics of cell processing and early development stage of the project (Table 5).

So it is the aim of this symposium today to find a way for the academe to conduct early phase drug development of seed compounds originated by the academe, that is, we are allowed to control also the early phase development to produce clinical grade compound or well-controlled cell processing according to phase 1 cGMP guideline. So I think iGMP or phase 1 cGMP plus IND registered system are the appropriate regulations for translational cell therapy.

COMMENTS / Q & A

Larghero: I would just like to add a comment: in France, we have to observe two regulatory rules. The first is regulation that is applied for any clinical trial. The other one is a specific regulation for cell therapy products that ask a full description, for each trial, of the cell therapy product (which cell types are present in the product, what are the controls performed during its production, what are the results of pre clinical studies, what are all the medium, growth factors and raw materials used for the production, and so on) and of the cell therapy unit by itself (description of the unit, plan of the lab, controls performed to ensure the safety of the persons and of the product......). Thus cell therapy activities are probably the most regulated field of medical research.

Table 5　iGMP+IND

iGMP + IND

appropriate regulations for Translational Cell Therapy

Simple is best !

ORIGINAL ARTICLE

Mismatch of minor histocompatibility antigen contributes to a graft-versus-leukemia effect rather than to acute GVHD, resulting in long-term survival after HLA-identical stem cell transplantation in Japan

T Katagiri[1], S Shiobara[2], S Nakao[3], M Wakano[1], E Muranaka[1], N Kuba[2], T Furukawa[4], J Tsukada[5], H Takeda[6], Y Aizawa[7] and M Harada[8]

[1]Faculty of Health Science, Kanazawa University School of Medicine, Kanazawa City, Japan; [2]Division of Transfusion Medicine, Kanazawa University Hospital, Kanazawa City, Japan; [3]Cellular Transplantation Biology, Kanazawa University Graduate School of Medical Science, Kanazawa City, Japan; [4]Division of Bone Marrow Transplantation, Niigata University Medical and Dental Hospital, Niigata City, Japan; [5]First Department of Internal Medicine, University of Occupational and Environment Health, Kitakyuhsyu City, Japan; [6]Medical Laboratory Division, Niigata University Medical and Dental Hospital, Niigata City, Japan; [7]Division of Hematology, Niigata University Graduate School of Medical and Dental Science, Niigata City, Japan and [8]First Department of Internal Medicine, Kyushu University School of Medicine, Fukuoka City, Japan

We determined the alleles of five polymorphic molecules including HA-1 and four adhesion molecules for 106 patients transplanted with HLA-identical stem cell grafts and investigated the association of mismatches as correlates of relapse and graft-versus-host disease (GVHD). All 106 recipients underwent stem cell transplantation (SCT) after myeloablative conditioning between 1985 and 2002. Risk status of disease at SCT was standard ($n = 63$) and high ($n = 42$). After SCT, 36, 49 and 33 developed acute GVHD, chronic GVHD and relapsed, respectively. Our patients relapsed at rates of 16.7 and 38.6% with one or more and without incompatibilities ($P = 0.013$). The relapse rates of patients with CD62L, CD31 codon 563, CD31 codon 125, HA-1 and CD49b incompatibilities were 5.9, 11.8, 15.4, 16.0 and 33.3%, respectively. The frequency of acute GVHD did not differ regardless of incompatibilities. In standard-risk group, the accumulated relapse rates of 19 and 44 patients with and without minor histocompatibility antigen incompatibility were 22% and unexpectedly 66%, respectively ($P = 0.02$). The probability of 12-year survival was 88% in the former and 66% in the latter patients ($P = 0.03$). Our data suggest that incompatibility of CD62L, CD31 codon 563 and CD31 codon 125 contributes to a graft-versus-leukemia effect rather than to GVHD, resulting in prolonged survival after HLA-identical SCT.
Bone Marrow Transplantation (2006) 38, 681–686. doi:10.1038/sj.bmt.1705506; published online 18 September 2006

Introduction

Donor-derived T lymphocytes that cause graft-versus-host disease (GVHD) might also induce graft-versus-leukemia (GVL) reactivity in an HLA-identical combination. Minor histocompatibility antigens (mHags) that induce GVHD are potential candidates for a GVL effect after allogeneic stem cell transplantation (SCT).[1-5]

HA-1 is an established mHag that was discovered by Goulmy et al.[1,2] and polymorphic adhesion molecules including CD31, CD49b and CD62L are immunodominant mHags that contribute to acute GVHD (a-GVHD) in Japanese[6] and Caucasian[7-9] populations.

As GVL effects are similar in both the populations, whereas the incidence and severity of a-GVHD is low among Japanese after allogeneic SCT and donor leukocyte infusion (DLI),[10,11] the roles of mHags in Japanese patients might differ from those of Western SCT patients. We therefore investigated the association of mHag mismatches with a-GVHD, as well as relapse and survival rates.

Materials and methods

Patients

During May 1998, 2002 and 2004, we collected various samples from donors and recipients before myeloablative SCT to analyze HA-1 and four polymorphic adhesion molecules. Patients underwent the procedure at Kanazawa University Hospital, Niigata University Medical and Dental Hospital and the University Hospital of Occupation

and Environment Health. Patients transplanted before May 1998 were also enrolled in this study. Peripheral blood cells from patients after SCT were of donor origin, and nail or buccal membrane samples were collected as host cells. Primary physicians were asked to report three times regarding the outcomes of SCT. All patients were followed up for at least 2 years after SCT.

Data included patient age, sex, diagnosis, stage of disease at transplantation, donor sex, date of transplant, conditioning regimen, GVHD prophylaxis, severity of acute and chronic GVHD, other major complications after transplantation, time of relapse, HLA serologically identified antigens and allele type in 35 out of the 106 patients. For patients with CML, type of relapse (molecular, cytogenetic or hematological) during stable, accelerated or blast phases was included. Treatment-related information included DLI, chemotherapy and α-interferon therapy. Treatment outcome was assessed according to the severity of GVHD and the response of leukemia to DLI. Survival, morbidity and recurrence of leukemia were also evaluated. Table 1 shows the characteristics of 65 male and 41 female patients who were transplanted with stem cells from 60 HLA-A, -B, -DR matched related donors and from 46 HLA-A, -B, -DR matched unrelated donors, where five of the 60 and 30 of the 46 were allele type-compatible donors.

Conditioning regimens consisted of 12 Gy fractionated TBI as 3 Gy × 4 or 2 Gy × 6 in 99 of 106 patients and seven of the 106 patients received a non-TBI regimen. Stem cell sources were BM (88), PBSC (15) and both (three). Post transplant immunosuppression consisted of short-term MTX + CyA (98), short-term MTX + FK (five) and CyA alone (three). None of the patients received T-cell-depleted marrow.

Table 1 describes patients' characteristics. Patients at standard risk were defined as those transplanted at first complete remission of acute leukemia, in chronic phase of chronic myeloid leukemia and refractory anemia of myelodysplastic syndrome.[12] High-risk patients consisted of those who were not assessed as being of standard risk. Patients with complications included 36 who developed a-GVHD (≥2), 53 who developed chronic GVHD and 33 who relapsed after allogeneic SCT.

Methods

Samples. The Institutional Review Board of Kanazawa University Hospital has approved the use of DNA analysis for mHag typing. We obtained written, informed consent of patients and donors to obtain DNA from their peripheral blood samples before transplantation. Peripheral blood and nail or buccal membranes were obtained from post transplant patients. The former was used as donor cells, and nail or buccal membrane, as host cells.

Allele typing of HA-1 and four polymorphic adhesion molecules. Alleles of HA-1 and four polymorphic molecules were typed as described by Maruya et al.[6] Briefly, purified genomic DNA for HA-1 and the polymorphic adhesion molecules were amplified by PCR using sequence-specific primers (Table 2). Amplification proceeded in 50 μl of PCR buffer (Applied Biosystems, Foster City, CA,

Table 1 Characteristics of mHag-incompatible and -compatible patients

		mHag compatibility	
		IC	C
All patients	106	36	70
Sex, n (%)			
Male	65	21 (58.3)	44 (62.9)
Female	41	15 (40.5)	26 (37.1)
Diagnosis, n (%)			
ALL	27	7 (19.4)	20 (28.6)
AML	29	11 (29.7)	18 (25.7)
CML	34	9 (24.3)	25 (35.7)
HD	1	0 (0)	1 (1.42)
NHL	5	2 (5.40)	3 (4.29)
MDS	10	7 (18.9)	3 (4.29)
Source of BMT, n (%)			
Sibling	60	22 (61.1)	38 (54.2)
Unrelated donor	46	14 (37.8)	32 (45.7)
Source of stem cell, n (%)			
BM	88	29 (80.5)	59 (84.3)
PBSC	15	4 (10.8)	11 (15.7)
BM + PBSC	3	3 (8.10)	0 (0)
Risk at transplantation, n (%)			
Standard	63	19 (51.4)	44 (62.9)
High	42	17 (47.2)	25 (35.7)
Not evaluated	1	0 (0)	1 (1.42)
Complications, n (%)			
Relapse	33	6 (16.7)[a]	27 (38.6)
Acute GVHD (≥2)	35	12 (33.3)	23 (32.9)
Chronic GVHD	52	17 (47.2)	35 (50.0)
Direction of transplantation, n (%)			
Female to male	25	9 (25.0)	16 (22.9)
Other[b]	81	27 (73.0)	54 (77.1)
Time of transplantation, n (%)			
1985–1997	35	13 (36.1)	32 (45.7)
1998–2002	61	23 (62.2)	38 (54.3)
Immunosuppressant, n (%)			
Short-term MTX + CyA	98	33 (89.2)	65 (92.8)
Short-term MTX + FK	5	3 (8.10)	2 (2.86)
Other[b]	3	0 (0)	3 (4.29)
Irradiation, n (%)			
TBI	97	34 (94.4)	63 (90.0)
Non-TBI	9	2 (5.40)	7 (10.0)

Abbreviations: ALL = acute lymphoblastic leukemia; AML = acute myelogenous leukemia; C = compatible; CML = chronic myelogenous leukemia; CyA = cyclosporine; FK = tacrolimus hydrate; HD = Hodgkin's disease; IC = incompatible; MDS = myelodysplastic syndrome and MDS including four refractory anemia, four refractory anemia with excessive blasts, one refractory anemia with excessive blasts in transformation and two overt leukemia; mHag = minor histocompatibility antigen; MTX = methotrexate; NHL = non-Hodgkin's lymphoma; PSL = prednisolone; TBI = total body irradiation.
[a]Relapse rate of incompatible group was significantly lower than that of compatible group.
[b]Short-term MTX + PSL, CyA, MTX.

USA), containing 10 mmol/l Tris-HCl (pH 8.3), 50 mmol/l KCl, 1.5 mmol/l MgCl$_2$, 0.2 mmol/l, each of the four deoxyribonucleotides (Applied Biosystems, Foster City,