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49. 秦　艶、橋井則貴、川崎ナナ、山口照英：強陰イオン交換 HPLC を用いたヘパリンナトリウム確認試験及び限度試験に関する研究．日本薬学会第 129 年会（2009.3.京都）
50. 西村　健、大高真奈美、瀬川宏知、内田恵理子、塙(古田)美玲、豊田淑江、山口照英、中西真人：細胞質持続発現型 RNA ベクターの性質検討と医療応用に向けた研究．第 31 回日本分子生物学会年会・第 81 回日本生化学学会大会・合同大会（2008.12.9-12　神戸）
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53. 田邊思帆里、佐藤陽治、鈴木孝昌、欒　洋、鈴木和博、山口照英：ヒト骨髄由来間葉系幹細胞に関するゲノムプロファイリング．日本ケミカルバイオロジー研究会第 3 回年会（2008.5.19）
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3．その他・政策提言

1. ICH 見解 「生殖細胞への遺伝子治療用ベクターの意図しない組み込みリスクに対応するための基本的な考え方」 平成１８年１０月
2. 「血液製剤のウイルスに対する安全性確保を目的とした核酸増幅検査(NAT)の実施に関するガイドライン」平成１６年８月３日（薬食発第 0803002 号）
3. 医薬発１３１４号改定 「ヒト（自己）由来細胞・組織加工医薬品等の品質及び安全性の確保に関する指針」作成（平成２０年２月８日）
4. 医薬発１３１４号改定「ヒト（同種）由来細胞・組織加工医薬品等の品質及び安全性の確保に関する指針」作成（平成２０年９月１２日）（薬食発第 0912006 号）
5. 薬食監麻発第 0327027 号「ヒト（自己）由来細胞・組織化更衣薬品等の製造管理・品質管理の考え方について」作成
6. 「生物由来原料基準」平成 17 年厚生労働省告示第 262 号
7. 「生物由来原料基準」平成 17 年厚生労働省告示第 177 号

G. 知的所有権の取得状況

１．特許取得

1. 佐藤陽治、山口照英、長谷川哲也、細野哲司、佐藤光利　特願 2006-109854「細胞の心筋細胞分化活性検出用マーカー」
2. 川崎ナナ、橋井則貴、山口照英　特願 2007-322161「同位体標識法と LC/MSn を利用した糖鎖比較定量法」

２．実用新案登録

なし

３．その他

なし

表1．HEV検出に用いたプライマー及びプローブ

HEV　1　(J. Virol. Methods 131, 65 (2006))

Forward: JVHEVF:　5'-GGTGGTTTCTGGGGTGAC-3'

Reverse: JVHEVR: 5'-AGGGGTTGGTTGGATGAA-3'

Probe: JVHEVP: 5'-FAM-TGATTCTCAGCCCTTCGC-BHQ1-3'

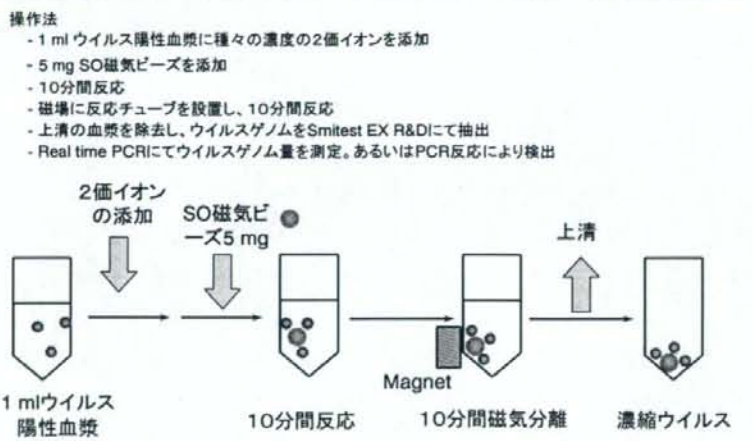
HEV　2　(J. Medical Virol. 78, 1076 (2006)を一部改変)

Forward: HEV-F:5'-GGCCGG(T/C)CAGCCGTCTGG-3'　　T/C=Y

Reverse: HEV-R:5'-CTGAGAATCAACCC(G/T)GTCAC-3'　G/T=K

Probe:HEV-P:5'-FAM-CGGT(G/A)CCGGCGGTGGTTTCT-TAMRA-3'　G/A=R

図1．2価イオンとスルホン酸(SO)磁気ビーズによるウイルス濃縮操作



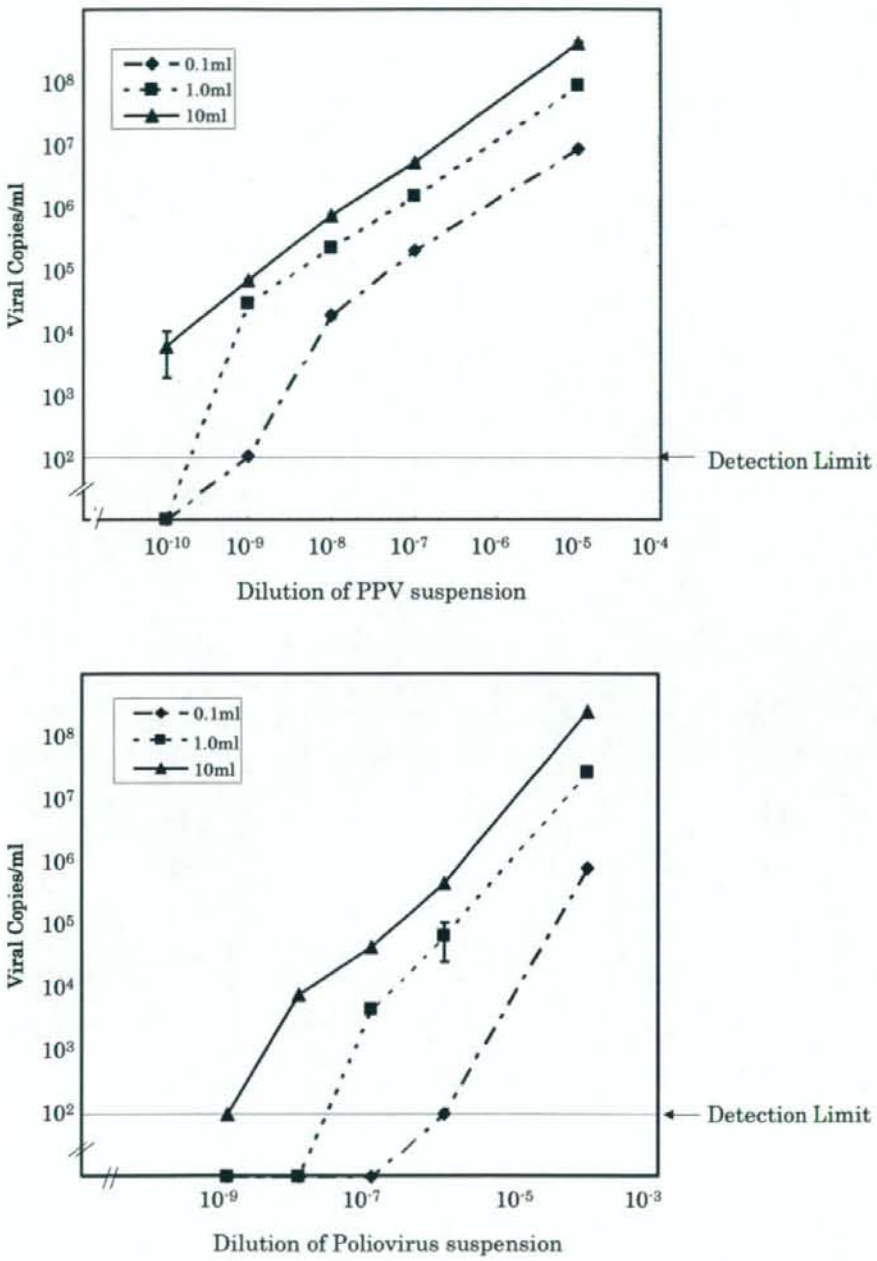


図2．PPV やポリオウイルスの Zn イオンと SO 磁気ビーズを組み合わせて濃縮.

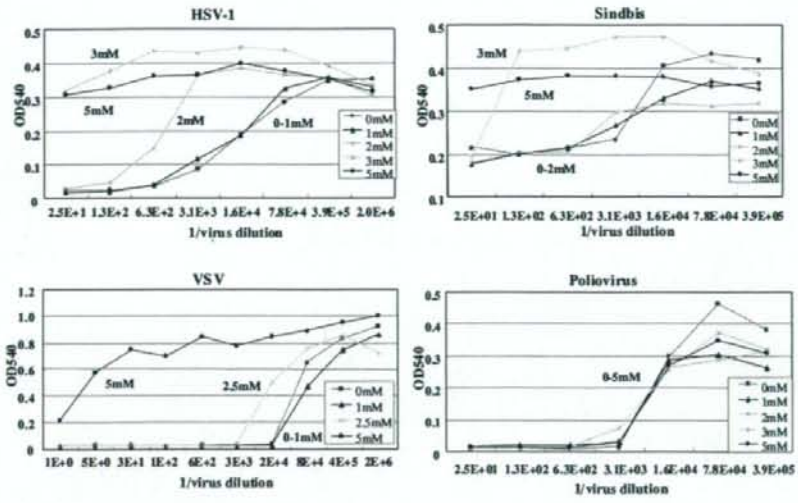


図3. PFOAによるウイルス不活化の濃度依存性

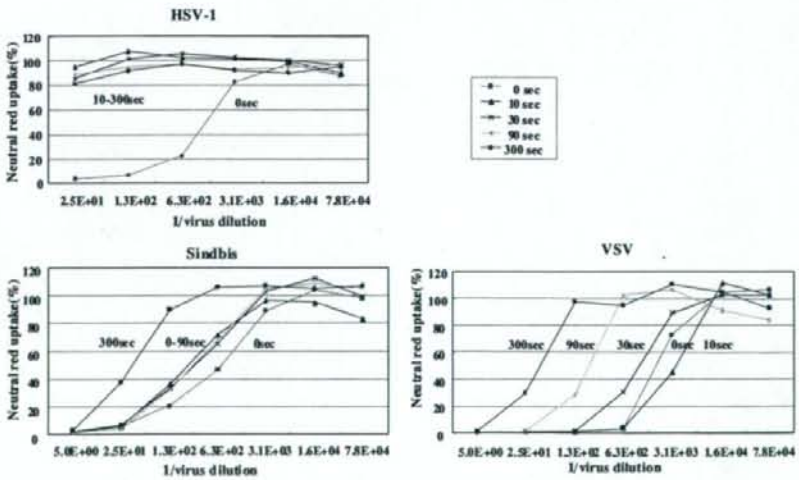


図4. PFOAによるウイルス不活化の時間依存性

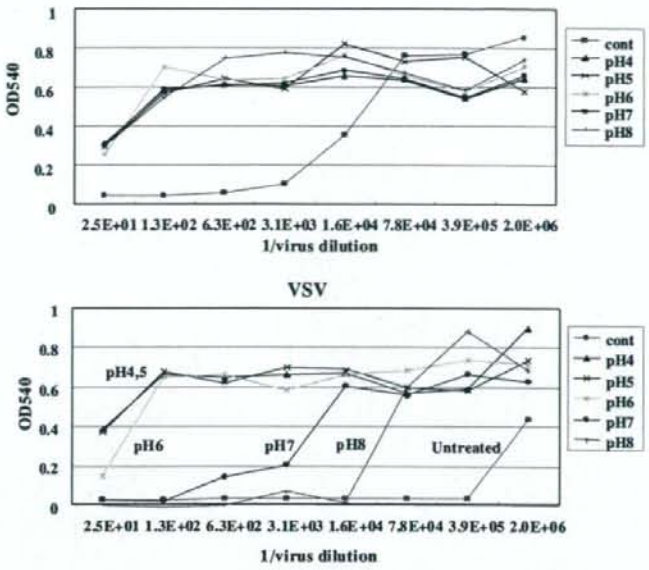


図５．PFOAによるウイルス不活化pHの影響

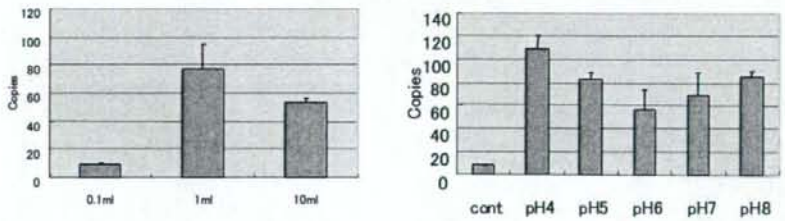


図6. PEI磁気ビーズを用いたHEVの濃縮と濃縮時のpH依存性

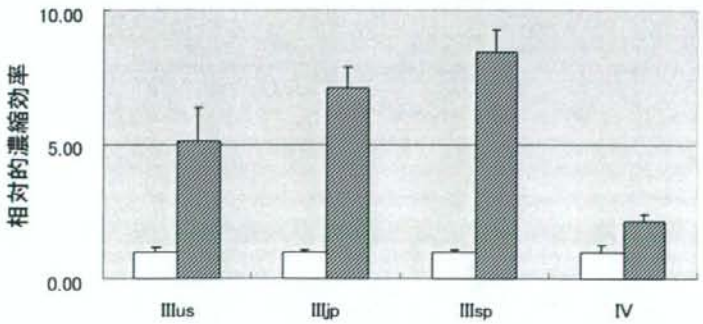


図7.　HEV各サブタイプの濃縮効率(白:濃縮前、斜線:濃縮後)

表2．－欧州・WHO・日本のガイドライン比較－

	欧　州 [1]	WHO [2]	日　本 [3], [4]
製造工程に含めるべきウイルス不活化／除去に「有効」＊な工程の数	原則として、機序の異なるものを2工程	機序の異なるものを2工程　（エンベロープウイルス）	原則として、機序の異なるものを2工程以上（但し、「有効」の定義は無し）
	このうち少なくとも2工程は非エンベロープウイルスに有効なもの	このうち少なくとも2工程は非エンベロープウイルスに有効なもの	－
	－	このうち少なくとも1工程は不活化工程	－
総 RF 値の許容範囲	エンベロープウイルスに対して原則8以上（「有効」＊工程×2）	エンベロープウイルスに対して8以上（「有効」＊工程×2）	－
	－	－	HIV, HBV, HCV 各モデルウイルスに対して9以上
	非エンベロープウイルスに対して原則4以上（「有効」＊工程×1）	非エンベロープウイルスに対して4以上（「有効」＊工程×1）	－
	原材料をはじめ、製造工程で混入し得ると見積もられるウイルス量より明らかに大	原材料中に最大存在し得ると見積もられるウイルス量よりかなり大きいことが原則	原材料中に含まれる可能性のある全てのウイルスを念頭において評価
ウイルスバリデーション評価に際して含めるべきウイルスの種類	・HIV-1 ・HBV/HSV モデルウイルス（例：PRV） ・HCV モデルウイルス（例：BVDV） ・HAV/B19 モデルウイルス（例：動物パルボウイルス）	原材料中に存在する可能性のあるウイルス（HIV、HBV、HCV（及び HAV、B19））に類似したもの	DNA 又は RNA、エンベロープの有無、粒子径の大小を考慮し、さらに物理的処理及び化学的処理に対する抵抗性が高いものを選択。 3 種類程度のモデルウイルスを組み合わせることが原則

＊RF 値に関しては、4以上であること

1) CPMP, "NOTE FOR GUIDANCE ON PLASMA-DERIVED MEDICINAL PRODUCTS" (CPMP/BWP/269/95 rev.3, 2001.1/25) & "NOTE FOR GUIDANCE ON ASSESSING THE RISK FOR VIRUS TRANSMISSION－NEW CHAPTER 6 OF THE NOTE FOR GUIDANCE ON PLASMA-DERIVED MEDICINAL PRODUCTS (CPMP/BWP/269/95)"（CPMP/BWP/5180/03, 2004. 10 / 21）

2) WHO EXPERT COMMITTEE ON BIOLOGICAL STANDARIZATION（Geneva, 2001. 11 / 26～30）

"Guidelines on Viral Inactivation and Removal Procedures Intended to Assure the Viral Safety of Human Blood Plasma Products"（2003. 1）

3）厚生省医薬安全局長，「血漿分画製剤のウイルスに対する安全性確保に関するガイドラインについて」（医薬発第 1047 号, 1999.8/30）

4）厚生労働省医薬食品局審査管理課長，同安全対策課長，同監視指導・麻薬対策課長，同血液対策課長，「血漿分画製剤のウイルス安全対策について」（薬食審査発第 1107001 号・薬食安発第 1107001 号・薬食監発第 1107001 号・薬食血発第 1107001 号, 2003.11/7）

表３．米国との比較

	米　国[1]	参考）日本ガイドライン[2], [3]
製造工程に含めるべきウイルス不活化／除去に「有効」＊な工程の数	機序の異なるものを２工程以上	原則として、機序の異なるものを２工程以上（但し、「有効」の定義は無し）
	このうち少なくとも１工程は非エンベロープウイルスに有効なもの	－
	このうち少なくとも１工程は不活化工程	－
総 RF 値の許容範囲	エンベロープウイルスに対して８以上（「有効」＊工程×２）	
	HIV、HBV モデルウイルス及び HCV モデルウイルスに対して１０以上	HIV, HBV, HCV 各モデルウイルスに対して９以上
	非エンベロープウイルスに対して６以上（「有効」＊工程×１）	－
	原材料中に最大存在し得ると見積もられるウイルス量の３～５log 以上であることが原則	原材料中に含まれる可能性のある全てのウイルスを念頭において評価
ウイルスバリデーション評価に際して含めるべきウイルスの種類	・HIV（技術的に可能なかぎり） ・HBV モデルウイルス ・HCV モデルウイルス	DNA 又は RNA、エンベロープの有無、粒子径の大小を考慮し、さらに物理的処理及び化学的処理に対する抵抗性が高いものを選択。３種類程度のモデルウイルスを組み合わせることが原則

＊RF 値に関しては、４以上であること

1) OBRR/CBER, Transmissable Spongiform Encephalopathies Advisory Committee（2002.6/26, 2003.2/20）& Blood Product Advisory Committee（2003.9/18）

2）厚生省医薬安全局長，「血漿分画製剤のウイルスに対する安全性確保に関するガイドラインについて」（医薬発第 1047 号, 1999.8/30）

3）厚生労働省医薬食品局審査管理課長，同安全対策課長，同監視指導・麻薬対策課長，同血液対策課長，「血漿分画製剤のウイルス安全対策について」（薬食審査発第 1107001 号・薬食安発第 1107001 号・薬食監発第 1107001 号・薬食血発第 1107001 号, 2003.11/7）

研究成果の刊行に関する一覧表

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A New Role of Thrombopoietin Enhancing *ex Vivo* Expansion of Endothelial Precursor Cells Derived from AC133-positive Cells*

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We previously reported that CD31^{bright} cells, which were sorted from cultured AC133$^+$ cells of adult peripheral blood cells, differentiated more efficiently into endothelial cells than CD31$^+$ cells or CD31$^-$ cells, suggesting that CD31^{bright} cells may be endothelial precursor cells. In this study, we found that CD31^{bright} cells have a strong ability to release cytokines. The mixture of vascular endothelial growth factor (VEGF), thrombopoietin (TPO), and stem cell factor stimulated *ex vivo* expansion of the total cell number from cultured AC133$^+$ cells of adult peripheral blood cells and cord blood cells, resulting in incrementation of the adhesion cells, in which endothelial nitric oxide synthase and kinase insert domain-containing receptor were positive. Moreover, the mixture of VEGF and TPO increased the CD31^{bright} cell population when compared with VEGF alone or the mixture of VEGF and stem cell factor. These data suggest that TPO is an important growth factor that can promote endothelial precursor cells expansion *ex vivo*.

Neovascularization is an important adaptation to rescue tissue from critical ischemia. Postnatal blood vessel formation was formerly thought to be primarily due to the migration and proliferation of preexisting, fully differentiated endothelial cells, a process referred to as angiogenesis. Recent studies provide increasing evidence that circulating bone marrow-derived endothelial progenitor cells (EPCs)[2] contribute substantially to adult blood vessel formation (1–5). Cell therapy using EPCs is widely performed to rescue tissue damaged due to critical ischemia.

Although EPCs have been thought to be derived from many kinds of cells, cells characterized as CD34$^+$ (6), AC133$^+$ (7, 8), and CD14$^+$ (9) are also thought to differentiate to EPCs. The main role of EPCs has been thought to be the release of angiogenic factors such as interleukin-8 (IL-8), granulocyte colony-stimulating factor (G-CSF), hepatocyte growth factor, and vascular endothelial growth factor (VEGF) (9). To obtain a sufficient number of EPCs for the treatment may be very important in cell therapy for critical ischemia.

On the other hand, EPCs are mobilized from bone marrow by many substances such as G-CSF (10), granulocyte macrophage-colony stimulating factor (GM-CSF) (5), VEGF (3), erythropoietin (11–13), and statins (14, 15) *in vivo*. To get as many EPCs as possible without unduly burdening the patient, it is desirable to establish efficient expansion methods for EPCs *in vitro*.

Thrombopoietin (TPO), initially identified as the primary regulator of platelet production (16), plays an important and nonredundant role in the self-renewal of and expansion methods for hematopoietic stem cells (17–19). Recently, TPO has been found to exert a proangiogenic effect on cultured endothelial cells (20). The mechanism by which hematopoietic cytokines support revascularization *in vivo*, however, remains unknown. TPO has increased the number of colony-forming units-granulocyte-macrophage (21) and of burst-forming units-erythroid (22) *in vivo* and leads to a redistribution of colony-forming units-erythroid from marrow to spleen. Moreover, TPO acts in synergy with erythropoietin to increase the growth of burst-forming units-erythroid and the generation of colony-forming units-erythroid from marrow cells (21, 23, 24).

In our previous study (25), we isolated AC133$^+$ cells and examined their endothelial differentiation *in vitro*. CD31(PECAM-1)$^+$ and CD31^{bright} cells appeared at an early stage of the *in vitro* differentiation of AC133$^+$ cells, and CD31^{bright} cells derived from AC133$^+$ cells were identified as the precursors of endothelial cells because CD31^{bright} cells had differentiated more efficiently to endothelial cells than others. Therefore, we conclude that CD31^{bright} cells derived from AC133$^+$ cells possess the typical character of EPCs. In this study, we analyzed the effects of TPO on the appearance of CD31^{bright} cells from AC133$^+$ cells, and we show that TPO plays an important role in *in vitro* EPC expansion.

EXPERIMENTAL PROCEDURES

Reagents—Recombinant TPO and recombinant stem cell factor (SCF) were kindly provided by Kirin-Amgen Inc. (Thousand Oaks, CA). Recombinant human VEGF was purchased from Strathmann Biotec AG (Hamburg, Germany). The AC133

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[2] The abbreviations used are: EPCs, endothelial precursor cells; VEGF, vascular endothelial growth factor; FN, fibronectin; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; PE, phycoerythrin; TPO, thrombopoietin; SCF, stem cell factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage-colony stimulating factor; IL, interleukin; PI3K, phosphatidylinositol 3-kinase; VEcad, vascular endothelial cadherin; eNOS, endothelial nitric oxide synthase; FBS, fetal bovine serum; STAT, signal transducers and activators of transcription; JAK, Janus kinase; KDR, kinase insert domain-containing receptor.

magnetic cell sorting kit and phycoerythrin (PE)-conjugated anti-CD133/2 antibody were from Miltenyi Biotec (Gladbach, Germany). Allophycocyanin-conjugated anti-CD110 (TPO receptor) antibody, fluorescein isothiocyanate (FITC)-conjugated anti-CD31 monoclonal antibody, FITC-conjugated anti-CD34 monoclonal antibody, and anti-STAT3 monoclonal antibody were from Pharmingen. Phycoerythrin-conjugated vascular endothelial cadherin (VEcad/CD144) antibody was from Beckman Coulter (Marseilles, France). Anti-vascular endothelial growth factor receptor-2 (Flk-1/KDR) monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and anti-human endothelial nitric oxide synthase (eNOS) rabbit polyclonal antibody (Cayman Chemical, Ann Arbor, MI) were obtained. Anti-phospho-Akt (Ser-473) antibody, anti-Akt antibody, and anti-phospho-STAT3 (Tyr-705) antibody were from Cell Signaling Technology (Beverly, MA). Fibronectin (FN)- and type IV collagen-coated dishes were purchased from Iwaki Co., Tokyo, Japan. Phycoerythrin-conjugated anti-CD14 antibody was from DakoCytomation (Glostrup, Denmark).

Preparation of Peripheral Blood Mononuclear Cells—Human cord blood was kindly supplied by the Metro Tokyo Red Cross Cord Blood Bank (Tokyo, Japan) with informed consent. The buffy coat fraction was prepared from voluntary donated human blood of Saitama Red Cross of Japan (Saitama, Japan). The blood sample was diluted with phosphate-buffered saline (PBS) containing 2 mM EDTA and was loaded on a Lymphoprep™ tube (Axis-Shield PoC AS, Oslo Norway) (density = 1.077). After being centrifuged for 20 min 800 × *g* at 18 °C, mononuclear cells were collected and washed with sorting solution (PBS supplemented with 2 mM EDTA and 0.5% bovine serum albumin).

Flow Cytometric Analysis of AC133 and CD34 Expression in Mononuclear Cells—To eliminate the dead cells, dead cells were stained with 7-amino actinomycin D. Mononuclear cells were labeled with PE-conjugated anti-AC133 monoclonal antibody and FITC-conjugated anti-CD34 monoclonal antibody simultaneously at 4 °C for 30 min. After washing with the sorting solution, flow cytometric analysis was performed with a FACSCalibur (BD Biosciences).

Magnetic Cell Sorting of AC133$^+$ Cells—Mononuclear cells were labeled with magnetic bead-conjugated anti-AC133 antibodies according to the protocol directed by the manufacturer. After the brief wash with the sorting solution, the cells were separated by a magnetic cell separator (autoMACS, Miltenyi Biotec, Gladbach, Germany), and the positive cells were then collected.

Culture of AC133$^+$ Cells—Isolated AC133$^+$ cells were cultured in EBM-2 (Cambrex Corp., East Rutherford, NJ) medium containing 20% heat-inactivated FBS and 30 mg/liter kanamycin sulfate at 37 °C under moisturized air containing 5% CO_2 with 50 ng/ml VEGF as control medium. Control medium containing VEGF was added with TPO, SCF, or both. Cells were plated on FN- or type IV collagen-coated dishes at a cell density of ~10^6 cells/ml. We have previously shown that EPCs can tightly adhere to an FN-coated dish but weakly to type IV collagen-coated dish (25). Analysis of adherent EPCs was performed on FN-coated dish and that of suspended EPCs on type IV collagen-coated dish. Half of the medium was exchanged once every 3–4 days with fresh medium. Adherent cells on FN-coated dish were fixed with ethanol chilled to −20 °C and then subsequently subjected to an immunostaining procedure or other treatments. Cells on type IV collagen-coated dish were subsequently subjected to flow cytometric analysis.

Immunostaining of Adherent Cells—After fixation with chilled ethanol (−20 °C), the cell layer was washed three times with PBS. Cells were incubated with 1% bovine serum albumin in PBS (−) for 1 h at 4 °C for blocking and then with each first antibody in 1% bovine serum albumin in PBS (−) for 1 h at 4 °C. After washing with PBS, the cells were incubated with FITC-conjugated anti-mouse IgG antibody or rhodamine-conjugated anti-rabbit IgG antibody for 1 h at 4 °C. Cells were washed with PBS and then examined using a Zeiss LSM 510 microscope with an excitation wavelength of 488 nm and an emission of 530/30 nm for FITC or 570/30 nm for rhodamine.

In every experiment, we used nonspecific immunoglobulin corresponding to the first antibody species as a control and confirmed that the cells were not stained with control immunoglobulin. The fluorescence intensity of 20 randomly selected cells was calculated using the Scion Image program within the linear range for quantitation.

Analysis of Cytokines in the Supernatant of CD31^{bright} and CD31$^+$ Cells—The expression of CD31 on cultured AC133$^+$ cells was determined with a flow cytometer. After AC133$^+$ cells were cultured for several days on either FN-coated or collagen type IV-coated dishes, both adherent and nonadherent cells were collected. The collected cells were labeled with FITC-labeled anti-CD31 antibody for 15 min at 4 °C. After a brief wash with 0.5% bovine serum albumin in PBS, flow cytometric analysis was performed. CD31^{bright} and CD31$^+$ cells were sorted from cultured AC133$^+$ cells with FACSAria (BD Biosciences). Sorted cells of both populations were subsequently cultured in EBM-2 supplemented with 20% FBS in the absence of any cytokines. After 5 days, the collected supernatant of cells was frozen at −20 °C. Cytokines were measured by a BD™ cytometric beads array Flex set system (BD Biosciences) according to the manufacturer's protocol.

Flow Cytometric Analysis of Various Cell Surface Markers in Cultured AC133$^+$ Cells—After AC133$^+$ cells were cultured for the indicated period, cells were co-stained with FITC-labeled anti-CD31 antibody and PE-labeled anti-CD14 antibody or PE-labeled VEcad antibody. Cells were also stained with FITC-labeled anti-CD31 antibody, allophycocyanin-labeled anti-CD110 antibody, and PE-labeled anti-AC133 antibody triply and then subjected to flow cytometry. Dead cells were eliminated by staining with 7-amino actinomycin D.

Calculation of the Absolute Number of CD31^{bright} Cells—The absolute number of CD31^{bright} cells was multiplied by the total cell number of each well, and the ratio of CD31^{bright} cells was analyzed by fluorescence-activated cell sorter.

Preparation of Cell Lysates and Immunoblotting—After cell sorting, AC133$^+$ cells were suspended in 20% FBS-EBM2 and cultured for 3 days in the presence of VEGF and TPO. Cells were collected and incubated in 2% FBS-EBM2 for 1 h. Cells were stimulated by 50 ng/ml TPO, 50 ng/ml VEGF, or both for 15 min. Cells (1×10^6) were collected and lysed in lysis buffer containing 1% Triton X-100, 10 mM K_2HPO_4/KH_2PO_4 (pH

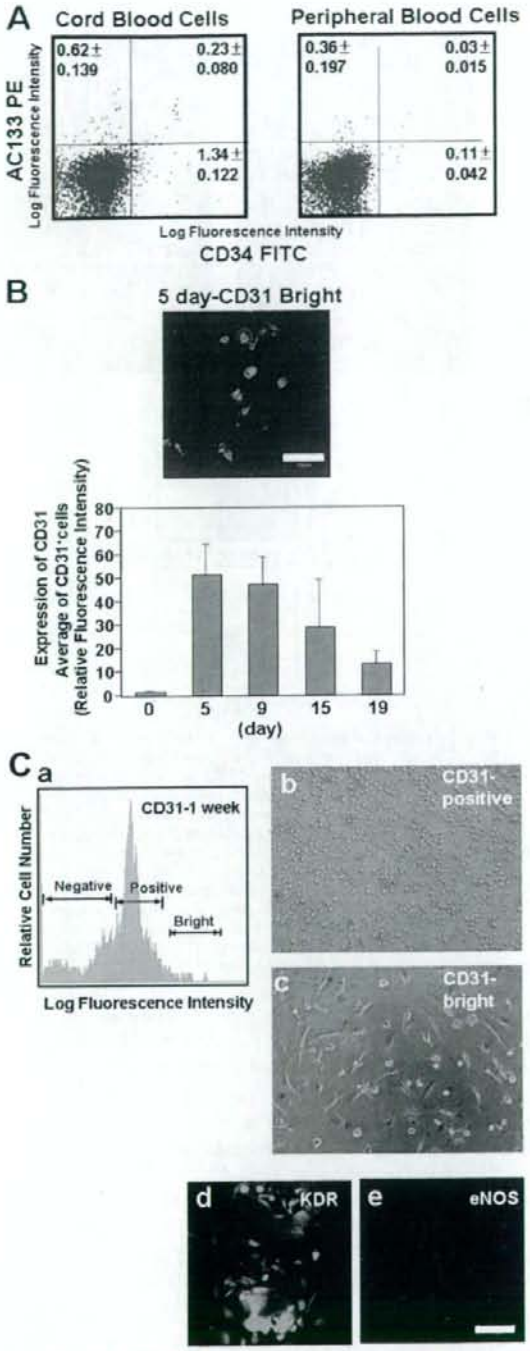


FIGURE 1. ***In vitro* differentiation of AC133$^+$ cells of cord blood into endothelial cells.** *A*, expression of AC133 and CD34 cells in human cord blood and peripheral blood mononuclear cells was analyzed by staining with AC133-PE (*vertical axis*) and CD34-FITC (*horizontal axis*). The *numbers* in the flow cytometric dot blots indicates the percentage of each population ± S.D. *B*, when AC133$^+$ cells were cultured for 19 days in the presence of VEGF on FN-coated dishes, the appearance of CD31$^+$ cells was analyzed. The *upper panel* shows the fluorescent photomicrograph of adhesion cells stained with FITC-conjugated anti-CD31 antibody after a 5-day culture. Quantitation of the fluorescence intensity of 20 CD31-positive cells was analyzed as described under "Experimental Procedures." Columns and bars represent the means ± S.D. from 20 cells (*B*, *lower panel*). *C*, the CD31-negative, positive, and bright cell populations prepared after 1-week cultivation of AC133$^+$ cells are shown in a representative histogram stained with FITC-conjugated anti-CD31 antibody. The *x* axis represents the log fluorescence intensity of CD31-FITC, *y* axis relative cell number (*panel a*). *Panels b* and *c* show phase-contrast microscopic photographs of cultured CD31-positive and bright cells, respectively, subsequently cultured for 1 week after cell sorting. The *bottom panels d* and *e* show the fluorescent photomicrographs of adhesion cells from the CD31^{bright} fraction stained with anti-KDR antibody and anti-eNOS antibody, respectively. *Scale bar*, 100 μm.

7.5), 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, and 50 mM β-glycerophosphate, along with 1/100 (v/v) protease inhibitor mixture (Sigma) and 1/100 (v/v) phosphatase inhibitor mixture (Sigma). The cellular lysate of 5×10^5 cells/lane was subjected to Western blotting analysis.

Statistical Analysis—Statistical analysis was performed using the unpaired Student's *t* test, and the dose response of TPO was compared between the four groups by one-way analysis of variance and the Tukey test using Prism 4 software. Values of $p < 0.05$ were considered to indicate statistical significance. Each experiment was repeated three times, and the representative data are indicated.

RESULTS

We previously reported that during the *in vitro* differentiation of peripheral blood AC133$^+$ cells into the endothelial cells, the expression of CD31 was the earliest marker among all of the tested markers (25). Moreover, by analyzing the ability of differentiation into endothelial cells, CD31^{bright} cells were shown to exhibit EPC character when compared with the CD31$^+$ fraction. Since cord blood is a rich source of blood stem cells such as CD34$^+$ and AC133$^+$ cells, it is expected to be a useful source for CD31^{bright} cells. At first, we attempted to determine whether the CD31^{bright} fraction derived from cord blood AC133$^+$ cells contained EPCs. As shown in Fig. 1*A*, the populations of AC133$^+$ CD34$^-$ cells, AC133$^-$ CD34$^+$ cells, and AC133$^+$ CD34$^+$ cells in cord blood were approximately four times greater than those in peripheral blood (Fig. 1*A*). After 5 days of cultivation of AC133$^+$ cells on an FN-coated dish, adherent CD31-positive cells were observed (Fig. 1*B*, *upper panel*). Analysis of the fluorescence intensity of CD31-positive cells revealed that the average fluorescence intensity in CD31$^+$ cells was highest on day 5 (Fig. 1*B*, *lower panel*), corresponding to the results of peripheral blood cells.

After 1 week of cultivation of AC133$^+$ cells on a collagen type IV-coated dish, on which cells adhered more loosely when compared with the FN-coated dish, cells were collected and sorted into CD31$^+$ and CD31^{bright} fractions, as shown in Fig. 1*C*, *panel a*, and both cell types were cultured on an FN-coated dish for 1 week after the sorting. The number of cells adhering and spreading was higher in the CD31^{bright} fraction (Fig. 1*C*, *panel c*) than in the CD31$^+$ fraction (Fig. 1*C*, *panel b*), and these adhering cells are apparently KDR- (Fig. 1*C*, *panel d*) and eNOS-positive (Fig. 1*C*, *panel e*). The large areas of intense green fluorescence represent the colonies of CD31^{bright} cells. These data indicate that CD31^{bright} cells derived from AC133$^+$ cells of both peripheral blood and cord blood are EPCs.

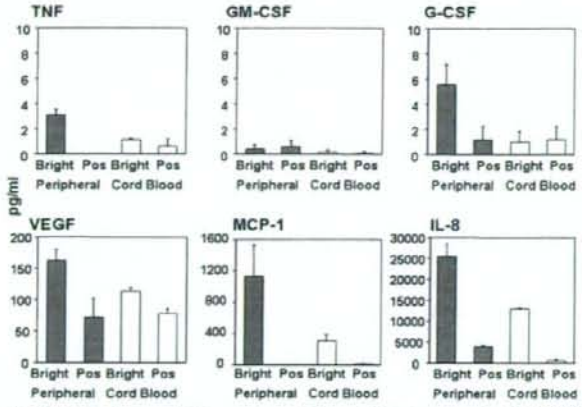


FIGURE 2. **Various cytokines released from CD31$^+$ cells and CD31^{bright} cells.** Production of various cytokines from CD31$^+$ cells and CD31^{bright} cells derived from AC133$^+$ cells cultivated for 5 days was measured. *Gray columns* indicate the cytokine production by cells from peripheral blood and open columns from cord blood. Columns and bars represent the means ± S.D. from three separate experiments. *TNF*, tumor necrosis factor; *Pos*, positive; *MCP-1*, monocyte chemoattractant protein-1.

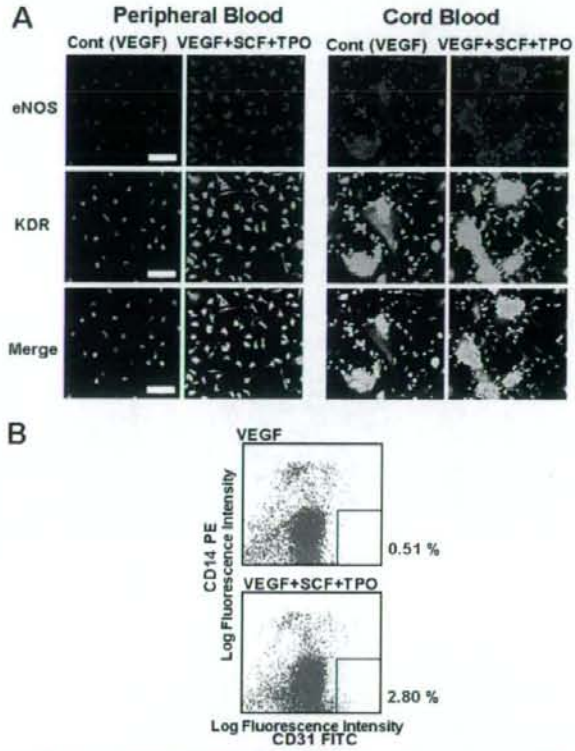
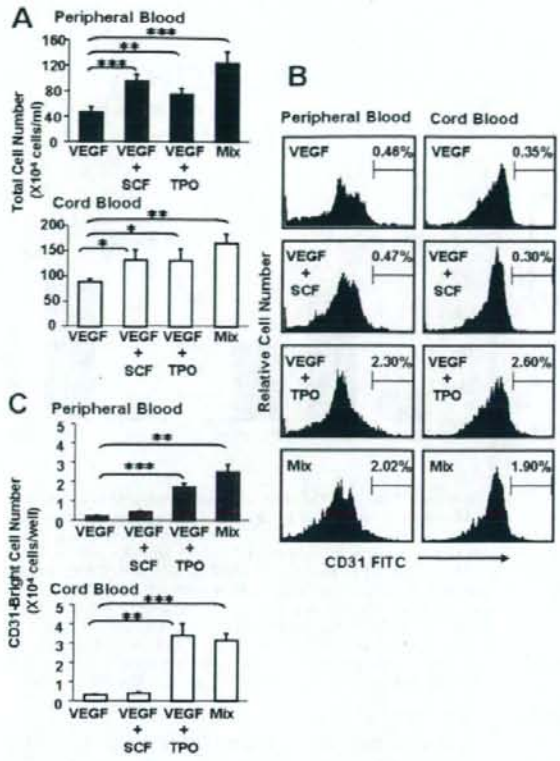


FIGURE 3. **Increment of EPCs from AC133$^+$ cells in the presence of TPO and SCF.** *A*, AC133$^+$ cells were differentiated for 2 weeks in the presence of either VEGF alone or the combination of TPO, SCF, and VEGF on an FN-coated dish. The *upper* and *middle panels* indicate the fluorescent photomicrographs of cells stained with anti-eNOS antibody and anti-KDR antibody, respectively. The *bottom panels* indicate the merged images of both antibodies. From the *left side*, control (*Cont*) and the mixture of peripheral blood, control, and the mixture of cord blood. *Scale bar*, 100 μm. *B*, CD14 and CD31 expression in cultured AC133$^+$ cells for 1 week was stained with CD14-PE (*vertical axis*) and CD31-FITC (*horizontal axis*). The *upper panel* indicates cells treated with VEGF alone, and the *lower panel* indicates cells treated with the mixture of VEGF, SCF, and TPO. The *number* on the *right side* of the flow cytometric dot blot indicates the percentage of the CD14$^-$ CD31^{bright} population.

Several reports have shown that EPCs produce cytokines (9, 26, 27), but the ability of CD31$^+$ or CD31^{bright} cells derived from AC133$^+$ cells to produce cytokines is not known. After cell sorting, quantitative analysis of cytokines released by CD31$^+$ cells and CD31^{bright} cells was carried out at 5 days after the cultivation. As shown in Fig. 2, IL-8 was markedly produced by CD31^{bright} cells from both peripheral blood and cord blood when compared with CD31$^+$ cells. The production of monocyte chemoattractant protein-1 (MCP-1) by CD31^{bright} cells was also higher than that of CD31$^+$ cells. The production of VEGF was higher by CD31^{bright} cells than by CD31$^+$ cells but not significantly. The production of all cytokines by CD31^{bright} cells from peripheral blood was higher than that from cord blood. Tumor necrosis factor-α, GM-CSF, and G-CSF were hardly produced by CD31^{bright} and CD31$^+$ cells. These data indicate that CD31^{bright} cells derived from AC133$^+$ cells have a strong ability to produce chemokines.

It has been reported that TPO and SCF are potent stimulators of multipotent cell proliferation (17, 19). Next, the effects of both growth factors on EPC growth and differentiation in our culture system were determined. After the addition of both TPO and SCF for 2 weeks, the expression of eNOS and KDR in adhered cells was analyzed (Fig. 3*A*). Fig. 3*A* clearly indicates that AC133$^+$ cells from both peripheral blood and cord blood differentiate into eNOS$^+$ and KDR$^+$ cells more efficiently in the presence of the mixture of TPO, SCF, and VEGF than of VEGF alone. Flow cytometric analysis revealed that the ratio of CD31^{bright} CD14$^-$ cells increased in the presence of the mixture of TPO, SCF, and VEGF when AC133$^+$ cells were cultured on collagen type IV-coated dish for 1 week (Fig. 3*B*).

We next examined which growth factor is dominant in the induction and proliferation of CD31^{bright} cells. The total cell number of cultured AC133$^+$ cells from both peripheral blood (Fig. 4*A*, *upper panel*) and cord blood (Fig. 4*A*, *lower panel*) significantly increased in the presence of TPO, SCF, or both growth factors when compared with that of VEGF alone during a 1-week period. As shown in Fig. 4*B*, however, the increment in the ratio of the CD31^{bright} cell population was observed only in the presence of TPO. The absolute number of CD31^{bright} cells, calculated by the total cell number and the ratio of the CD31^{bright} cell population, was markedly increased by TPO (Fig. 4*C*). In contrast, SCF induced the increase in total cell number to the same level as TPO (Fig. 4*A*), but it did not induce the increase in either the ratio of the CD31^{bright} cell population (Fig. 4*B*) or the number of CD31^{bright} cells (Fig. 4*C*). Next, we examined whether TPO and VEGF can synergistically affect the induction of CD31^{bright} cells during a 1-week cultivation. As shown in Fig. 4*D*, although VEGF had no effects on the total cell number (Fig. 4*D*, *panel a*), it increased the ratio of the CD31^{bright} cell population to 1.4-fold higher than that of the control (Fig. 4*D*, *panel b*), resulting in a slight increase in the number of CD31^{bright} cells (Fig. 4*D*, *panel c*). Thrombopoietin alone induced an increase in not only the total cell number (Fig. 4*D*, *panel a*) but also the ratio of the CD31^{bright} cell population (Fig. 4*D*, *panel b*), resulting in an ~24-fold increment of the absolute



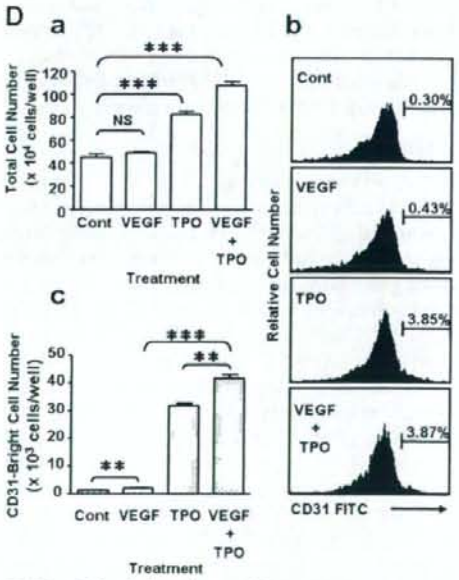


FIGURE 4. **Stimulative effects of TPO on induction of CD31^{bright} cells.** *A*, alteration of the cell number of cultivated AC133$^+$ cells for 1 week in the combination of growth factors. *Mix*, VEGF + SCF + TPO. *B*, the flow cytometric histogram of AC133$^+$-derived cells stained with FITC-labeled anti-CD31 antibody after a 1-week culture. The *representing number* in the flow cytometric histogram indicates the percentage of the CD31^{bright} cell population. The *left panels* are peripheral blood, and the *right panels* are cord blood. *C*, CD31^{bright} cells numbers were calculated by both the total cell number and the ratio the of CD31^{bright} population. *D*, the effects of TPO alone on EPC differentiation derived from AC133$^+$ cells of cord blood. The *upper left panel* (*a*) shows the total cell number after a 1-week culture, the *right panels* (*b*) show the flow cytometric histogram of AC133$^+$-derived cells stained with FITC-labeled anti-CD31 antibody, and the *lower left panel* (*c*) shows the calculated CD31^{bright} cell number. Columns and bars represent the means ± S.D. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). *NS*, not significant; *Cont*, control.

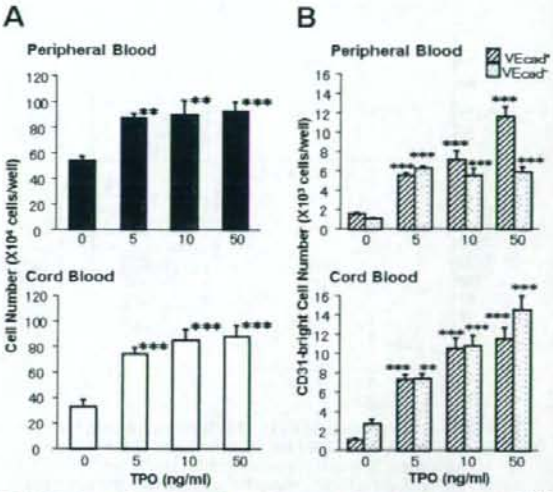


FIGURE 5. **Dose-dependent effects of TPO on the induction of CD31^{bright} cells from AC133$^+$ cells.** AC133$^+$ cells were treated with various concentrations of TPO for 1 week. The *left panels* (*A*) are the total cell number of cultured AC133$^+$ cells from peripheral blood (*upper panel*) and cord blood (*lower panel*). The *right panels* (*B*) are the calculated CD31^{bright} cell number from peripheral blood (*upper panel*) and cord blood (*lower panel*). Columns and bars represent the means ± S.D. (**, $p < 0.01$; ***, $p < 0.001$). *Striped* and *dotted columns* represent CD31^{bright}VEcad$^+$ cells and CD31^{bright}VEcad$^-$ cells, respectively.

number of CD31^{bright} cells when compared with the control (Fig. 4*D*, *panel c*). The concomitant treatment with both VEGF and TPO showed a synergic increase in the number of CD31^{bright} cells (Fig. 4*D*, *panel c*).

When AC133$^+$ cells were cultured with various concentrations of TPO in the presence of constant concentrations of VEGF (50 ng/ml), the total cell number from both peripheral blood (Fig. 5*A*, *upper panel*) and cord blood (Fig. 5*A*, *lower panel*) significantly increased at 5 ng/ml of TPO when compared with the control, and there was no significant difference in the total cell number from 5 to 50 ng/ml of TPO. However, TPO increased the ratio of CD31^{bright} cells of flow cytometry dose-dependently as follows: control, 0.50%; 5 ng/ml, 1.36%; 10 ng, 1.42%; 50 ng/ml 1.90% in peripheral blood and control, 1.16%; 5 ng/ml, 1.99%; 10 ng, 2.51%; 50 ng/ml 2.96% in cord blood. TPO markedly induced the differentiation of AC133$^+$ cells into CD31^{bright}VEcad$^+$ cells in the case of both peripheral blood (Fig. 5*B*, *upper panel*) and cord blood (Fig. 5*B*, *lower panel*) in a dose-dependent manner. In the case of cord blood cells, differentiation into CD31^{bright}VEcad$^-$ cells was also induced by TPO.

The effects of TPO on total cell number during 6-day culture of AC133$^+$ cells were determined. Although the total cell num-

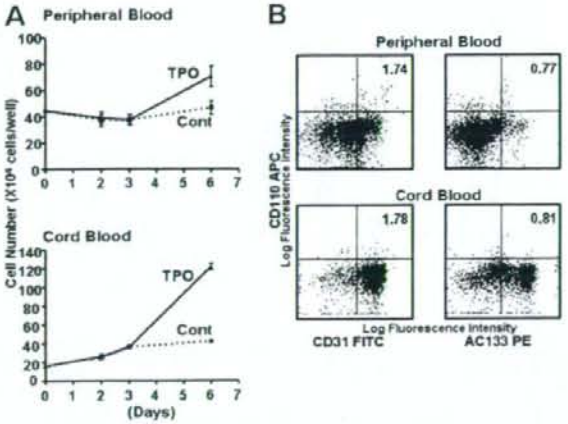


FIGURE 6. **Time-course analysis of TPO-treated AC133$^+$ cells and expression of TPO receptor (CD110).** *A*, alteration of cell number was counted at 2, 3, and 6 days. *Solid* and *dotted lines* indicate TPO-treated cells and control (*Cont*) VEGF alone) cells, respectively. The results represent mean ± S.E. of triplicate wells. *B*, flow cytometric analysis of CD110 expression on AC133$^+$ cells cultured for 3 days was carried out. The *y axis* represents the log fluorescence intensity of CD110-allophycocyanin (*APC*), and the *x axis* represents that of CD31-FITC (*left panels*) and AC133-PE (*right panels*). The *number* in the flow cytometric dot blot indicates the percentage of CD110$^+$ CD31$^+$ and CD110$^+$ AC133$^+$ populations, respectively. The *upper panels* are peripheral blood, and the *lower panels* are cord blood.

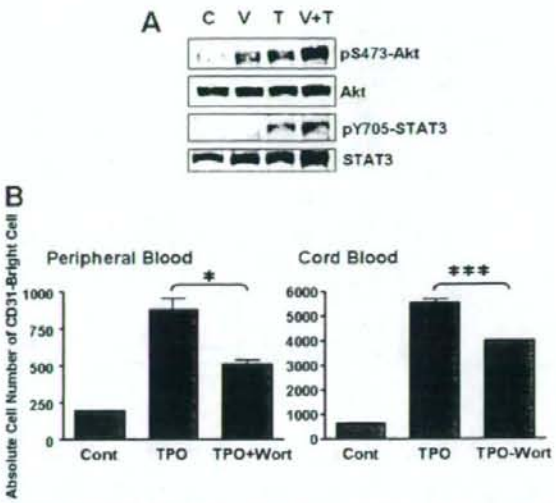


FIGURE 7. **Analysis of TPO-induced signal transduction on AC133$^+$ cells of cord blood.** *A*, activation of Akt or STAT3 was analyzed by Western blotting with anti-phospho-specific Ser-473-Akt antibody (*top panel*) and reprobed with anti-Akt antibody (*second panel*), or with anti-phospho-specific Tyr-705-STAT3 antibody (*third panel*) and reprobed with anti-STAT3 antibody (*lower panel*) after stimulation by VEGF, TPO, or both VEGF and TPO for 15 min using 3-day-cultured AC133$^+$ cells. *C*, control; *V*, VEGF; *T*, TPO. *B*, the effects of wortmannin on CD31^{bright} cell induction were investigated. The *right panel* shows peripheral blood, and the *left panel* shows cord blood. The *y axis* represents the CD31^{bright} cell number. *Wort*, 100 nM wortmannin. Columns and bars represent the means ± S.E. (*, $p < 0.05$; ***, $p < 0.001$). *Cont*, control.

ber from AC133$^+$ cells slightly and constantly increased from day 0 to day 6 in the absence of TPO, total cells markedly increased after the third day in the presence of TPO (Fig. 6*A*). Next, the alternation of TPO receptor (CD110) expression was analyzed during the cultivation of AC133$^+$ cells. Although the percentages of both AC133$^+$ CD110$^+$ cells and CD31$^+$ CD110$^+$ cells were 0% just after magnetic cell sorting, 3 days after the cultivation, ~2% of CD31$^+$ CD110$^+$ cells (Fig. 6*B*, *left panel*) and 1% of AC133$^+$ CD110$^+$ cells (Fig. 6*B*, *right panel*) appeared from AC133$^+$ cells in the peripheral blood and cord blood, respectively. These data indicate the possibility that sorted AC133$^+$ cells may differentiate into AC133$^+$ CD110$^+$ cells and may subsequently proliferate and differentiate into EPCs in response to TPO.

It has been reported that TPO activates the PI3K/Akt pathway (28) or JAK/STAT pathway (20, 29, 30) in target cells. In addition, in the present study, TPO induced a marked proliferation of AC133$^+$ cells after 3-day culture, and CD110 expression in cells cultured for 3 days from both cord blood and peripheral blood was also observed (Fig. 6, *A* and *B*). We then attempted to determine whether TPO activates Akt or STAT in AC133$^+$ cells cultured for 3 days by analyzing the phosphorylation at Ser-473 of Akt or the phosphorylation at Tyr-705 of STAT3, which are the active forms of Akt or STAT3, respectively. As shown in Fig. 7*A*, phosphorylation at Ser-473 of Akt was stimulated by both VEGF and TPO at 15 min and was more markedly stimulated by concomitant treatment with VEGF and TPO than by a single treatment (Fig. 7*A*, *top panel*). Phosphorylation at Tyr-705 of STAT3 was observed only in the presence of TPO, and unlike in the phosphorylation at Ser-473 of Akt, an increased amount of phosphorylation was not observed in the concomitant presence of VEGF and TPO (Fig. 7*A*, *third panel*). On the other hand, there was no difference in the expression of Akt and STAT3 protein levels (Fig. 7*A*, *second panel* and *bottom panel*, respectively). The induction of CD31^{bright} cells was not perfectly but significantly inhibited by wortmannin, an inhibitor of PI3K, suggesting that the PI3K/Akt pathway plays an important role in TPO-induced EPC differentiation (Fig. 7*B*).

DISCUSSION

We have previously reported that CD31^{bright} cells derived from AC133$^+$ cells in human peripheral blood are EPCs (25). In the present study, CD31^{bright} cells also appeared from AC133$^+$ cells prepared from cord blood, which are a rich source of stem cells during the early period of cultivation (Fig. 1, *A* and *B*). When cells were separated in terms of CD31 expression (Fig. 1*C*), CD31^{bright} cells differentiated into KDR-positive and eNOS-positive adherent cells. These data indicate that CD31^{bright} cells derived from AC133$^+$ cells in cord blood have some characteristics similar to those of EPCs in peripheral blood. Although these EPCs in both cord blood and peripheral blood could not form tube-like structure by themselves on Matrigel (data not shown), they secreted angiogenic growth factors (Fig. 2) such as VEGF, IL-8 (31, 32), and monocyte chemoattractant protein-1 (MCP-1) (33). It has been reported that there are at least two types of EPCs: early EPCs and late EPCs. Early EPCs are unable to form tube-like structures and secrete VEGF and IL-8 showing peak growth at 2–3 weeks (9, 26, 27). Late EPCs with the ability to proliferate and having a cobblestone shape appear late at 2–3 weeks, show exponential growth at 4–8 weeks, and have the ability to form tube-like structures

(26, 27, 34). Rehman *et al.* (9) have reported that EPCs derived from monocytes/macrophages do not proliferate but instead release potent proangiogenic growth factors. In many studies (9, 26, 27, 35–37), because the origin of early EPCs was $CD14^+$ cells or was not precluded by monocytic cells, CD14 expression was still observed in the EPCs after cultivation. In our study, in which $AC133^+$ cells were used as the origin of the EPCs, CD14 expression was not observed in $CD31^{bright}$ cells induced by TPO (Fig. 3*B*). Although the $CD31^{bright}$ cells identified as EPCs in this report and in a previous report did not correspond to their cells in terms of the origin of the cells or cell surface markers, these cells may be early EPCs that can release potent proangiogenic growth factors (Fig. 2). In any event, EPCs are thought to be a heterogeneous population, unlike late EPCs, which have a high ability to proliferate.

Circulating EPCs are up-regulated under physiological or pathological conditions and also by 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors (14, 15) and cytokines such as erythropoietin (11–13) and G-CSF (10). In this report, we have revealed the possibility of marked expansion of EPCs *in vitro* by TPO. Brizzi *et al.* (20) have reported that TPO directly stimulates endothelial cell motility and neoangiogenesis. In the present study, TPO may have played a stimulatory role in the differentiation of EPCs from circulating stem cells.

Although both TPO and SCF have the same potency with regard to proliferation of $AC133^+$ cells (Fig. 4*A*), TPO specifically induces an increase in the ratio of the $CD31^{bright}$ cell population when compared with SCF (Fig. 4, *B* and *C*). To develop useful cell therapy products for severe ischemia, it has been considered desirable to establish the efficient expansion of EPCs *in vitro*. Thrombopoietin could increase $CD31^{bright}$ cells (EPCs) even in the absence of VEGF. Kirito *et al.* (38) have reported that TPO enhances expression of VEGF in hematopoietic cells through induction of hypoxia-inducible factor 1α. These observations suggest the possibility that the production of EPCs by TPO may be supported by VEGF produced by $AC133^+$ cells. However, from the perspective that TPO and VEGF have synergistic effects on the induction of EPCs, TPO seems to induce EPCs through another signaling cascade.

Thrombopoietin is a major regulator of the proliferation, differentiation, and maturation of megakaryocytes (39, 40). The results from recent studies suggest that TPO can act not only as a lineage-specific hematopoietic growth factor but also can affect other hematopoietic cell types. For example, TPO alone does not induce proliferation of long term repopulating hematopoietic stem cells. However, in combination with SCF or IL-3, TPO has several synergistic effects on cell proliferation (19). Our results have revealed a new role of TPO in the production of EPCs.

In the process of differentiation of $AC133^+$ cells into $CD31^{bright}$ cells, both peripheral blood and cord blood appear to be very similar. $AC133^+$ cells of cord blood, however, have a stronger ability to proliferate than those of peripheral blood (Fig. 6*A*). Moreover, TPO stimulates the induction of $CD31^{bright}VEcad^-$ cells only from cord blood (Fig. 5*B*) at high concentrations. Hur *et al.* (26) have reported that $VEcad^-$ EPCs are thought to be an early EPC. It is therefore thought that $AC133^+$ cells of cord blood are more immature than those of peripheral blood.

Although the total cell number treated with TPO slightly increased in a dose-dependent manner (Fig. 5*A*), the $CD31^{bright}$ cell number markedly increased as the TPO concentration increased (Fig. 5*B*). These data suggest the possibility that a higher concentration of TPO may be needed for $CD31^{bright}$ cell induction from $AC133^+$ cells.

When $AC133^+$ cells were stimulated by TPO or VEGF, an increase in the phosphorylation of Akt at Ser-473 was observed. This increase was strongly enhanced by concomitant treatment with VEGF and TPO (Fig. 7*A*). The induction of $CD31^{bright}$ cells by these growth factors (Fig. 4*D*) was consistent with the increase in the phosphorylation of Akt at Ser-473. TPO but not VEGF could also stimulate the phosphorylation of STAT3 at Tyr-705. We previously reported that the PI3K/p70 S6 kinase pathway and the JAK/STAT3 pathway were important for proliferation and differentiation, respectively, in neutrophilic differentiation (41, 42). Owing to the stimulation of both the PI3K/Akt and the JAK/STAT pathways, we postulated that TPO may be a stronger stimulator of EPC production than VEGF. As shown in Fig. 7*B*, however, wortmannin could not completely inhibit the induction of $CD31^{bright}$ cells. Therefore, a pathway other than the PI3K/Akt pathway may also work for the proliferation and differentiation of EPCs.

The observation of unfavorable angiogenesis has recently been reported after transplantation of bone marrow mononuclear cells in patients with thromboangiitis obliterans (43). Moreover, transfer of both spleen cell-derived EPCs and bone marrow mononuclear cells accelerate atherosclerosis in apoE knockout mice, whereas EPC transfer reduces markers associated with plaque stability (44). These observations suggest that transplantation of differentiated cells from EPCs may be useful therapy as regenerative medicine.

In conclusion, we have demonstrated a new role of TPO in enhancing the differentiation of $AC133^+$ cells into $CD31^{bright}$ cells (EPCs) *in vitro*. These findings may contribute to further development of cell therapy for critical ischemia.

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