TGN1412によるCD28占有率の算出

Data

Dose TGN1412 : 0.1 mg/kg

Body weight : 70 kg

Molecular weight TGN1412 : 150,000

Blood volume : 5L , Plasma volume : 2.5L

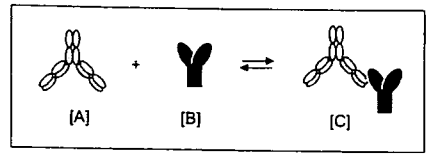
T lymphocyte count at baseline (before dosing) : 1.3 x 10^9 cells per L blood

CD28 receptors per T cell : 150,000 (Bryl et al. J. Immunol. 167, 3231, 2001)

Kd : 1.88 nM (TeGenero, information in public domain)

Binding affinity (Kd) = [A][B]/[C]
[A] = free mAb
[B] = free ligand
[C] = mAB-ligand complex

AB/C = 1.88



Calculation

Total TGN1412 concentration in plasma immediately post-dosing
= 0.1 x 70 / 150,000 / 2.5 x 10^6 = 18.7 nM (A + C)

Total ligand (CD28) concentration exposed to plasma at baseline
= 1.3 x 10^9 x 150,000 (receptors/cell) x 5 / N_A x 1/2.5 x 10^9 = 0.648 nM (B + C)

⇨ Drug-ligand concentration (C) immediately post-dosing = 0.587 nM

⇨ Percentage CD28 receptors occupied by TGN1412 = 90.6%

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図9

許容される標的占有率

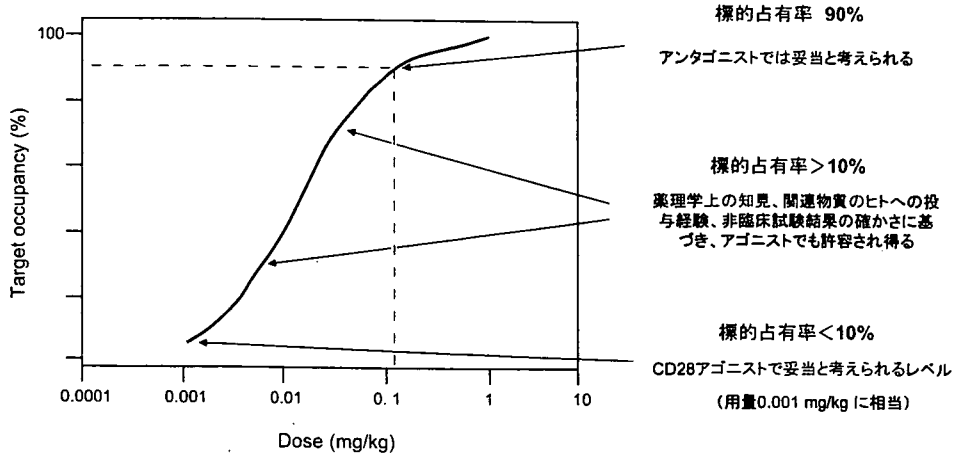

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図10

図9のように標的占有率を求めるためには、標的分子の濃度が既知でなければならないが、標的分子が組織や、容量が一定でない滑液などに存在する場合は、それは容易ではない。そこで一般的に、抗体の標的分子の濃度が投与された抗体の濃度より十分低いと仮定できる場合は、以下の式により標的占有率を概算できるとされている（図11：図中の解釈は本稿の著者によるもの）[4]。

$$Ro = 1 / (1 + Kd\,[nM] / (187\,[nM/mg/kg] \times Dose\,[mg/kg]))$$

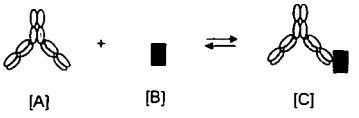
例えば、Kd[nM]の200分の1の値に対応する量[mg/kg]を投与された場合、初期の標的占有率は約50％となる。

標的占有率の概算

抗体の濃度が標的分子（受容体）の濃度より十分高いと考えられる場合は、用量と結合定数から、以下の式により標的占有率を概算することができる。

$$\text{Fractional ligand occupancy (Ro)} = 1 / \left(1 + \frac{Kd\,[nM]}{187\,[nM/mg/kg] \times Dose\,[mg/kg]}\right)$$

Body weight : 70 kg
Molecular weight : 150,000
Blood volume : 5L , Plasma volume : 2.5L



AB/C = Kd

A+C = Dose [mg/kg] x 70 [kg] / 150,000 / 2.5 x 10^6
= 187 [nM/mg/kg] x Dose [mg/kg]

Ro = C / (C+B)
= 1 / (1+B/C)
= 1 / (1+Kd/A)
≒ 1 / (1+Kd/(A+C))
= 1 / (1+Kd/(187xDose))

A >> B のとき、A >> Cと考えられるので、A ≒ A+C

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図11

これらの結果から、TGN1412のMABELは0.001 mg/kg程度と考えられる。したがって、ヒト初回投与量として許容される上限は、NOAELを基準に求められた0.1 mg/kgと比較して低い方の用量である0.001 mg/kgであったと考えられる（図12）。

TGN1412のヒト初回投与量は、カニクイザルに投与された最高用量である50 mg/kgをNOAELとして算出されたが、IL-6などのサイトカイン放出が薬理作用か有害作用かの見極め次第で、この判断の妥当性が異なってくる。MABELを指標とする場合は、検出された作用が薬理作用であるか有害作用であるかの区別は不要であるので、薬理作用と有害作用の区別が難しい場合は、MABELを基準にすることで、適切な用量設定を行うことができると考えられる。

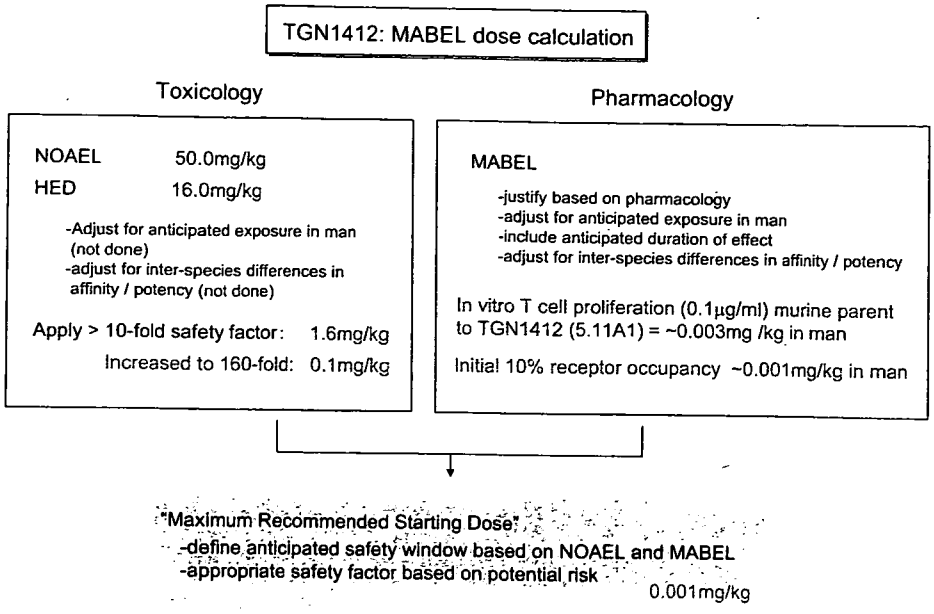

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図 12

1.4. TGN1412 事故が薬の開発（臨床・非臨床）に与えたインパクト：まとめ

TGN1412 の臨床試験は、リスクの高い医薬品の非臨床・臨床試験をどのように行うかを議論する契機となった。臨床における安全性確保のためには、ヒト初回投与試験で有害事象が生じるリスク要因の見極め、非臨床試験系の妥当性の検証、ヒト初回投与量の算出などに関して、個々の医薬品の開発段階での判断を適切に行うことが重要であると考えられる。TGN1412 の事故を受けて作成された EMEA のガイドラインは、非臨床試験から臨床試験への移行にあたって特段の注意を要するのはどのような医薬品か、また、ヒトで有害事象が発生するリスク要因があると考えられる医薬品の非臨床試験をどのように実施し、臨床試験をどのようにデザインするかを考える上で、今後の参考になるものと思われる。

本稿で考察した事項に関連して、TGN1412 事故を教訓に今後考えるべきこととして、以下のようなことが挙げられる。

(1) サイトカイン放出症候群に関して：

・リンパ球の活性化あるいはエフェクター細胞の活性化作用を持つ抗体医薬品では、常にサイトカイン放出症候群に対する十分な注意が必要である。

(2) 非臨床試験に関して：

・ヒトでの作用を予測し得るヒト細胞／組織を用いた試験系の開発を推進すべきである。

・ヒト由来細胞と動物由来細胞の反応性の比較を行うことは、非臨床試験系の妥当性を評価する上で有用であると思われる。

・TGN1412 と類似した作用機構を持つ抗体医薬品では、抗体のドライコーティングや血管内皮細胞との共培養系を用いてヒトリンパ球の反応を解析することにより、ヒトでの反応の予測に有用な知見が得られる可能性が考えられる。

・ヒトの組織・細胞パネルを用いた抗体医薬品の交差反応性の検証は、安全性に関する

重要な情報を与え得るため、非臨床試験の一つとして推奨されるものであると考えられる。

・目的外の作用も含めた医薬品の影響を評価するためには、トランスクリプトミクス、プロテオミクス、メタボロミクスなどのOmics解析も有用であると思われる。

(3) 新しいコンセプトに基づく医薬品の開発に関して：

・CD28のように複雑な作用を持つ分子を標的とするには、適切な用法用量の設定が必須であり、病態の理解を深め、個々の患者がどの状態にあるかを見極める診断方法の開発も必要であると思われる。

1.5. 今後の展望

TGN1412は、T細胞に発現するCD28を標的とするモノクローナル抗体であり、免疫調節作用により、関節リウマチやB細胞慢性白血病に用いることが考えられていた。その開発は前述のような結果に終わったが、抗体医薬品やその他のバイオ医薬品には、現在開発中のものも含めて、免疫制御を目的としたものが多い。これらの開発が進んだ結果、自己免疫疾患である関節リウマチのように、バイオ医薬品が治療に不可欠な存在となっている疾患もある。我が国においても、関節リウマチの治療に、キメラ型抗TNFα抗体Infliximab、あるいは可溶性TNF受容体とFcの融合タンパク質Etanerceptが使用できるようになり、既存の薬物療法では難しかった寛解を導くことも可能になったとされている[37]。その他に、ヒト抗TNFα抗体、抗IL-6受容体抗体、抗CD20抗体、抗RANKL抗体などの抗体医薬品や、CTLA4とFcの融合タンパク質なども関節リウマチの治療に有効であることが期待されており、関連領域で有用なバイオ医薬品は今後も増えると予想される。

2007年までに22品目のモノクローナル抗体が治療用医薬品として上市され（表5）、現在も多くの抗体医薬品の開発が精力的に進められている。その多くが免疫系に作用するものであるが、その他のバイオ医薬品にも、インターフェロン類、G-CSFやIL-2などのサイトカイン類のように、免疫系に作用するものが多い。これらは、高い標的特異性を持ち疾病治療に有用である一方で、その使用に伴い、TGN1412で生じたようなサイトカイン放出症候群のみならず、感染症や悪性腫瘍といった様々な有害事象が生じることも報告されている。免疫抑制作用を持つ医薬品では感染症が生じるリスクは避け得ないが、TNFα阻害薬で報告されているB型肝炎の再燃や結核のように、臨床で使用されて初めて明らかになる重篤なものもあることから、安全性評価には市販後調査なども含めた対策が必要と考えられる。また、免疫抑制作用を持つバイオ医薬品であるEfalizumab（抗CD11抗体）、Infliximab（抗TNFα抗体）、Adalimumab（抗TNFα抗体）、Etanercept（TNF受容体とFcの融合タンパク質）、Alefacept（LFA3とFcの融合タンパク質）、Abatacept（CTLA4とFcの融合タンパク質）では、いずれも悪性腫瘍の発生が報告されており、免疫抑制作用を持つバイオ医薬品では、悪性腫瘍の発生に常に注意が必要であろう。

現在、抗体医薬品の開発に多くの企業が注力している理由としては、これまでに抗体の医薬品としての価値が実証されてきたこと、また、抗体医薬品の開発の成功率が高いという近年の医薬品開発の実績があると思われる（米国において、第Ⅰ相臨床試験が行われたもののうち上市されたものの割合は、低分子化合物では約5％であるのに対して、抗体医薬品では約20％であると報告されている）[38]。抗体作製の技術革新もこの動きを後押ししており、ファージディスプレイ法やヒト抗体遺伝子導入マウスを用いてヒト抗体を作成する技術が開発され、さらに、低分子化あるいはバイスペシフィック化した抗体など、改変型の抗体の開発も進められている。抗体以外にも、融合タンパク質や、アミノ酸配列改変型、修飾構造改変型などの各種の改変型

表5

これまでに認可された抗体医薬品および融合タンパク質医薬品

名称	商品名	種類	構造	標的	主な適応疾患	承認年 US	承認年 EU	承認年 Japan
Muromonab-CD3	Olthoclone OKT3	マウス抗体	IgG2a	CD3	腎移植後の急性拒絶反応	1986	NA	1991
Abciximab	ReoPro	キメラ抗体	IgG1(Fab)	GPIIb/IIIa	心筋虚血	1994	NA	NA
Rituximab	Rituxan, MabThera	キメラ抗体	IgG1κ	CD20	B細胞性非ホジキンリンパ腫	1997	1998	2001
Daclizumab	Zenapax	ヒト化抗体	IgG1κ	CD25	腎移植後の急性拒絶反応	1997	1999	NA
Basiliximab	Simulect	キメラ抗体	IgG1κ	CD25	腎移植後の急性拒絶反応	1998	1998	2002
Palivizumab	Synagis	ヒト化抗体	IgG1κ	RSV F protein	RSウイルス感染	1998	1999	2002
Infliximab	Remicade	キメラ抗体	IgG1κ	TNFα	関節リウマチ	1998	1999	2002
Trastuzumab	Herceptin	ヒト化抗体	IgG1κ	HER2	転移性乳癌	1998	2000	2001
Gemtuzumab ozogamicin	Mylotarg	ヒト化抗体	IgG4κ(カリケアマイシン結合)	CD33	急性骨髄性白血病	2000	NA	2005
Alemtuzumab	Campath, MabCampath	ヒト化抗体	IgG1κ	CD52	B細胞性慢性リンパ性白血病	2001	2001	NA
Adalimumab	Humira	ヒト抗体	IgG1κ	TNFα	関節リウマチ	2002	2003	NA
Ibritumomab tiuxetan	Zevalin	マウス抗体	IgG1κ(Y-90標識)	CD20	B細胞性非ホジキンリンパ腫	2002	2004	NA
Omalizumab	Xolair	ヒト化抗体	IgG1κ	IgE	喘息	2003	2005	NA
Iodine 131 Tositumomab	Bexxar	マウス抗体	IgG2aλ(I-131標識)	CD20	非ホジキンリンパ腫	2003	NA	NA
Efalizumab	Raptiva	ヒト化抗体	IgG1κ	CD11	尋常性乾癬	2003	2004	NA
Cetuximab	Erbitux	キメラ抗体	IgG1κ	EGFR	頭頸部癌、結腸・直腸癌	2004	2004	NA
Bevacizumab	Avastin	ヒト化抗体	IgG1	VEGF	結腸・直腸癌	2004	2005	2007
Natalizumab	Tysabri	ヒト化抗体	IgG4κ	α4integrin	多発性硬化症	2004	2006	NA
Tocilizumab	Actemra	ヒト化抗体	IgG1	IL-6R	キャッスルマン病	NA	NA	2005
Ranibizumab	Lucentis	ヒト化抗体	IgG1κ(48Kフラグメント)	VEGF-A	加齢黄斑変性	2006	2007	NA
Panitumumab	Vectibix	ヒト抗体	IgG2κ	EGFR	結腸・直腸癌	2006	NA	NA
Eculizumab	Soliris	ヒト化抗体	IgG2/4κ	C5a	発作性夜間血色素尿症	2007	2007	NA
Denileukin Diftitox	Ontak	融合タンパク質	IL-2 + Diphtheria toxin	IL-2受容体	皮膚T細胞リンパ腫	1999	NA	NA
Etanercept	Enbrel	融合タンパク質	TNFR + Fc	TNF	関節リウマチ	1998	2000	2005
Alefacept	Amevive	融合タンパク質	LFA3 + Fc	CD2	尋常性乾癬	2003	NA	NA
Abatacept	Orencia	融合タンパク質	CTLA4 + Fc	CD80/CD86	関節リウマチ	2005	NA	NA

NA: not approved

Reichert JM et al. Nature Biotech. 23, 1073, 2005 をもとに作成

タンパク質性医薬品、さらにタンパク質性医薬品以外に視野を広げれば、siRNAなどの核酸医薬品、細胞組織利用医薬品、遺伝子治療薬の開発においても、実用化に向けた研究が進められており、構造の面でも作用の面でも新規性が高く、非臨床・臨床試験での評価が難しい医薬品は今後も増え続けると予想される。

臨床での安全性確保のため、新たな非臨床試験系の開発や、非臨床試験の妥当性検証を十分に行う努力が必要であることは言うまでもなく、本書で紹介されているような、毒性試験に追加されつつある新たな基礎技術などは、極めて有効な手段となるであろう。しかし、ヒトとモデル動物では生物学的にも生活環境の面でも違いがあることを考えると、非臨床試験ですべてを明らかにすることは困難である。バイオ医薬品の安全性確保の上で最も重要な懸念事項の一つであるヒトに対する免疫原性は、現状では臨床試験に加えて市販後の調査を経て評価されている。リスク低減のための対処法を考慮した上で、有用な医薬品開発にあたっての合理的な規制環境の整備が求められる。

1.6. おわりに

TGN1412の事故を教訓に、今後、主として医薬品の開発側には、臨床試験の安全性を担保するための新たな試験法の開発や、非臨床試験系の妥当性に関するより確実な検証が求められ、規制側には、被験者の安全を確保しつつ、新薬創出の妨げとならないよう、非臨床・臨床試験に求められる要件を整理していくことが望まれる。その際には、非臨床試験ですべてを明らかにすることは困難であること、医薬品のヒトにおける有効性・安全性は臨床試験を経て明らかにされるものであり、市販後調査なども含めた総合的な対応が必要であることを再確認しておきたい。

国立医薬品食品衛生研究所の生物薬品部／遺伝子細胞医薬部が事務局となっているバイオロジクスフォーラムでは、2003年以降、年1回の学術集会を開催して、バイオロジクスの品質・安全性確保に関して産官学の関係者による議論の場を設けている。また、医薬品医療機器総合機構では、2006年度から国際バイオロジクスフォーラムが開催され、バイオロジクスについての国際的な情報交換、あるいはバイオロジクスに関する適正な規制の在り方をめぐる共通の認識を深めるための場が提供されている。さらに、2007年には日本薬学会レギュラトリーサイエンス部会が主催する医薬品評価フォーラムが発足し、第1回の集会ではバイオ医薬品の開発と評価がテーマとして取り上げられた。このような議論を通じて医薬品開発の実情に則した規制環境が整備され、有用な新薬が安全かつ速やかに、社会に送り出されることが期待される。

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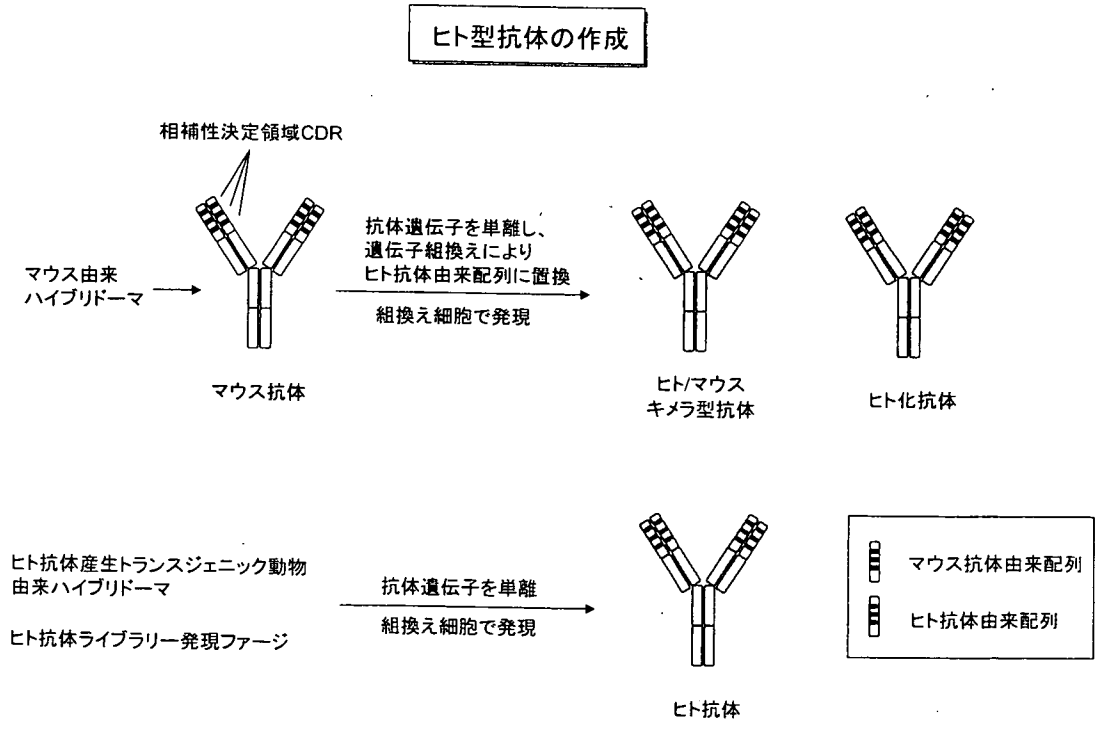
注1：バイオ医薬品

遺伝子組換え技術や細胞培養技術などのバイオテクノロジーを用いて製造される医薬品。組換えタンパク質医薬品、細胞培養医薬品、遺伝子治療薬、細胞治療薬等が含まれる。狭義には、バイオテクノロジーを応用して製造されるタンパク質性医薬品を指し、インスリン、成長ホルモン、エリスロポエチン、インターフェロン類、サイトカイン類、モノクローナル抗体などが代表例。

注2：ヒト化抗体

マウスに抗原を免疫して得た脾細胞とマウスミエローマを融合させたハイブリドーマから産生されるモノクローナル抗体はマウス抗体であるため、ヒトに投与すると、抗原性を示す、血中半減期が短い等の問題があり、医薬品としては適していない場合が多い。これらを解決するために、目的の抗体を産生するハイブリドーマから単離した抗体遺伝子を改変し、抗原決定に関与しない部分をヒト抗体に置き換える“抗体のヒト化技術”が開発された。マウス抗体遺伝子の不変領域をヒト抗体遺伝子に置換して作製した抗体を「キメラ型抗体」、不変領域に加えて、相補性決定領域（complementarity determining region：CDR）のみを残して可変領域もヒト抗体遺伝子に置換して作製した抗体を「ヒト化抗体」という（注2－図）。最



注2－図

近では、ヒト抗体遺伝子を導入したマウスやファージディスプレイ法を用いてヒトモノクローナル抗体を取得する技術が開発、実用化され、既に上市されたヒト抗体もある。

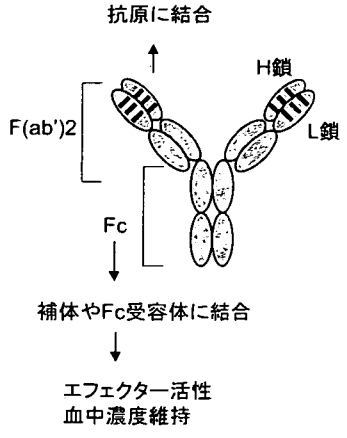
TGN1412の開発過程では、ラットCD28に対するマウスモノクローナル抗体JJ316が作製されて疾患モデルにおけるCD28アゴニスト抗体の有用性が明らかにされ、続いて、ヒトCD28に対するマウスモノクローナル抗体5.11A1が作製された。5.11A1抗体遺伝子の改変によりヒト化を行い、作製されたものがTGN1412、あるいは、そのIgG1バリアントTGN1112である。

注3：抗体の構造と機能

抗体は、相同な2本の重鎖（H鎖）と相同な2本の軽鎖（L鎖）がS-S結合で結ばれた構造を持つ。抗体の機能としては、第一義的には可変領域の抗原結合部位を介して抗原に結合することであり、さらに、Fc部分を介してFc受容体あるいは補体と結合し、抗体依存性細胞障害（Antibody Dependent Cellular Cytotoxicity: ADCC）や補体依存性細胞障害（Complement Dependent Cytotoxicity: CDC）により抗原を発現する細胞を障害するエフェクター機能を有している（注3－図）。

抗体には、IgG、IgA、IgM、IgD、IgEの5つのクラスが存在するが、これまでに医薬品として開発されたモノクローナル抗体は、すべてIgGである。ヒトIgGには、IgG1～4の4つのサブクラスが存在し、サブクラスごとにエフェクター機能等に差があることが知られている（Filpula D. *Biomol. Eng.* 24: 201, 2007）。組換え抗体医薬品では、目的とする医薬品の特性に応じたサブクラスが選択される。これまでに開発されている組換え抗体医薬品では、サブクラスがIgG1のものがほとんどであり、エフェクター機能により標的分子を発現する細胞を障害する効果が期待できる。IgG2はエフェクター機能が低い。IgG3はヒンジ領域が長いため他のサブクラスと比較して分子量が大きく、エフェクター機能は高い。凝集体を形成することがあるとされている。IgG4は補体結合能を持たないことが特徴である。生体内のIgG4では、重鎖＋軽鎖の交換が起こり、バイスペシフィックな分子にもなることが知られている（Aalberse RC et al., *Immunol.* 105: 9, 2002）。

抗体の構造と機能



IgG各サブクラスのFc受容体および補体との結合

		IgG1	IgG2	IgG3	IgG4
単球	FcγRI	++	−	+++	++
	FcγRIIa	+	(+)	++	−
	FcγRIIIa	+	−	+	−
好中球	FcγRIIa	+	−	+	−
	FcγRIIIb	+	−	+	−
補体		++	+	+++	−

＜多田富雄監訳　免疫学イラストレイテッドより改変＞

注3－図

注４：抗体医薬品の品質

抗体医薬品は、作製した抗体遺伝子をCHO細胞、あるいは、Sp2/0細胞、NS0細胞などに導入して細胞の培養上清中に抗体を分泌させ、その培養上清から目的の抗体を精製することにより生産する。（TGN1412はCHO細胞で製造された。）したがって、最終的に得られる抗体医薬品の品質（有効成分の構造・組成、物理化学的性質、免疫化学的性質、生物学的性質などの特性の他、目的物質由来不純物、製造工程由来不純物、混入汚染物質の存在等も含めて評価される）は、生産細胞の特性や培養条件、あるいは精製工程などの製造工程に大きく影響を受ける。そのため、抗体医薬品をはじめとするバイオ医薬品の品質・安全性確保においては、目的産物の特性解析を詳細に行い、望ましい有効性・安全性プロファイルの得られる目的物質の品質を規定する方法（規格および試験方法）を明らかにすると共に、製造ロットごとに品質の差が生じないよう、製造工程を厳密に管理することが重要である。

バイオ医薬品の品質・安全性確保に関しては、日米欧でこれまでに議論された結果が国際調和ガイドラインとしてまとめられている（注４－表（1））。我が国においては、バイオ医薬品の承認申請に際して申請者がどの程度のレベルでどの程度の量のデータを蓄積すればよいかなどを示すため、注４－表（2）のようなガイドラインが定められている。

注４－表（1）

バイオ医薬品の品質・安全性確保に関するICHガイドライン

Q5A　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
Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin
（2000.2.22）【厚生省医薬安全局審査管理課長　医薬審第329号】

Q5B　組換えDNAを応用したタンパク質生産に用いる細胞中の遺伝子発現構成体の分析
Quality of Biotechnological Products: Analysis of the Expression Construct in Cells Used for Production of R-DNA Derived Protein Products
（1998.1.6）【厚生省医薬安全局審査管理課長　医薬審第3号】

Q5C　生物薬品（バイオテクノロジー応用製品／生物起源由来製品）の安定性試験
Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products
（1998.1.6）【厚生省医薬安全局審査管理課長　医薬審第6号】

Q5D　生物薬品（バイオテクノロジー応用医薬品／生物起源由来医薬品）製造用細胞基剤の由来、調製及び特性解析
Derivation and Characterization of Cell Substrates Used for Production of Biotechnological/Biological Products
（2000.7.14）【厚生省医薬安全局審査管理課長　医薬審第873号】

Q5E　生物薬品（バイオテクノロジー応用医薬品／生物起源由来医薬品）の製造工程の変更にともなう同等性／同質性評価
Comparability of Biotechnological/Biological Products Subject to Changes in Their Manufacturing Process
（2005.4.26）【厚生労働省医薬食品局審査管理課長　薬食審査発第0426001号】

Q6B　生物薬品（バイオテクノロジー応用医薬品／生物起源由来医薬品）の規格及び試験方法の設定
Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products
（2001.5.1）【厚生労働省医薬局審査管理課長　医薬審第571号】

S6　バイオテクノロジー応用医薬品の非臨床における安全性評価
Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals
（2000.2.22）【厚生省医薬安全局審査管理課長　医薬審第326号】

国際合意に達したICHガイドラインの内容は、国内におけるバイオ医薬品の試験や評価を行う際の基本となり、そこに記載されている内容については遵守する必要がある。合意された各ICHガイドラインの内容については国内版が作成され、国内通知として出されている。

注４－表 (2)

我が国におけるバイオ医薬品関連ガイドライン

バイオ医薬品の承認申請に関して、申請者が、どの程度のレベルでどの程度の量のデータを蓄積すればよいかなどを示すために、我が国では以下の3つのガイドラインが公表されている。

(1) 薬審第243号通知(昭和59年3月30日)
「組換えDNA技術を応用して製造される医薬品の承認申請に必要な添付資料の作成について」

(2) 薬審1第10号通知(昭和63年6月6日)
「細胞培養技術を応用して製造される医薬品の承認申請に必要な添付資料の作成について」

(3) 都道府県衛生主管部(局)薬務主管課宛事務連絡(平成元年5月)
「薬審1第10号通知に関する質疑応答について」

注５：サイトカイン放出症候群に関する添付文書上の記載

医薬品添付文書においては、サイトカイン放出症候群は、“薬剤投与中または投与開始後24時間以内に多く現れる有害反応の総称”であるinfusion reactionに含めて記載されている場合が多い。例えばRituximabの場合は、infusion reactionとサイトカイン放出症候群を臨床上、厳密に区別することが困難であること、また、薬剤投与に伴って認められる有害事象に対して2つの異なった用語が用いられることによって対処方法が異なる印象を与え、臨床現場に混乱を来す可能性が懸念されるとして、infusion reaction

注５－表

サイトカイン放出症候群に関連する医薬品添付文書中の記載概要(抜粋)

	Muromonab-CD3 (抗CD3抗体)	Rituximab (抗CD20抗体)	Alemtuzumab (抗CD52抗体)
日本	【重要な基本的注意】 投与初期に発熱等のインフルエンザ様症状があらわれるので、その旨を患者にあらかじめ説明しておくこと。また、その間は患者を厳密に観察すること。	【重要な基本的注意】 本剤の初回投与中又は投与開始後24時間以内に多くあらわれるinfusion reactionが約90%の患者において報告されている。 【警告】 本剤の投与開始後30分～2時間よりあらわれるinfusion reactionのうちアナフィラキシー様症状、肺障害、心障害等の重篤な副作用により、死亡に至った例が報告されている。	未承認
US	【警告】サイトカイン放出症候群 ほとんどの患者で、活性化リンパ球または単球からのサイトカイン放出に起因する急性の臨床症状(サイトカイン放出症候群)が生じる。 【有害事象】サイトカイン放出症候群 腎移植の急性拒絶反応の治験で、最初の2回の投与後に、2%以下の患者で致死的なレベルの肺浮腫が報告されている。	【有害反応】Infusion reactions 大部分の患者で初回投与時に、発熱や悪寒/硬直などの軽度～中程度のinfusion reactionが生じた。 【枠付き警告】Fatal infusion reactions: 投与24時間以内の死亡例が報告されている。	【警告】Infusion-related events: 低血圧、硬直、発熱、気管支痙攣、悪寒などのinfusion-related eventsが生じている。 【枠付き警告】Infusion reactions:重篤な、時に致死的なinfusion reactionが生じ得る。
EU *	未承認	【投与方法】 サイトカイン放出症候群の発生に備え、患者を注意深くモニターすること。 【特別な警告と使用上の注意】 重篤なサイトカイン放出症候群が生じるリスクの高い腫瘍細胞数の多い患者では、他の治療法がない場合にのみ、特別な注意のもとに使用すること。	【副作用】Infusion-related reactions: 非常に一般的に報告された反応(サイトカイン放出に起因する)は、発熱、硬直、悪心、嘔吐等の急性のinfusion-related reactionsであった。 稀に、サイトカイン放出に伴って気管支痙攣、低酸素などの重篤な反応がおこり、致死的な結果となる例があった。

EU * :Summary of product characteristicsより抜粋。
いずれも、患者用添付文書ではinfusion reactionとしての記載のみ。

に統一されている（衛研発第2458号　審査報告書：http://211.132.8.246/shinyaku/g0106/04/38010100_21300AMY00273_110_1.pdf)。注5－表にMuromonab CD3、Rituximab、およびAlemtuzumabの日米欧における添付文書中のサイトカイン放出症候群に関連する表現を記載した。これらの医薬品では、サイトカイン放出症候群の発生を防ぐために、解熱薬や抗炎症薬の前投与が推奨されている。

Infusion reactionの原因としては、アナフィラキシー反応がよく知られているが、アナフィラキシー反応と比較して、サイトカイン放出症候群は抗体医薬品の初回投与時に起こる頻度が高いことが特徴である。これは、生体内の標的分子数が初回投与時に最も多く、2回目以降は初回に投与された抗体の効果により標的分子を発現する細胞が減っていることが原因であると考えられている。

☆コラム「生殖発生毒性の理解に役立つホームページ」コラム☆

最近は生殖発生毒性のみを専門にしている毒性研究者は少なくなりました。一般毒性も生殖発生毒性も兼任されている方や特殊毒性すべてを担当されている方もいます。若い方では実際に動物室でラット新生児の性別を見分けたり、胎児の骨格を染色して観察をした経験がなく、報告書の評価をされている人もいると聞きます。もちろん、教科書等でしっかり勉強されておられるのですが、現実感がわかないのも無理のないことかも知れません。

そんな専門ではないが、生殖発生毒性に関係しなくてはならなくなった方へ、

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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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Toshie Kanayasu-Toyoda[‡], Akiko Ishii-Watabe[‡], Takayoshi Suzuki[§], Tadashi Oshizawa[§], and Teruhide Yamaguchi[‡1]

From the Divisions of [‡]Biological Chemistry and Biologicals, and [§]Cellular and Gene Therapy Products, National Institute of Health Sciences, Kamiyoga 1-18-1, Setagayaku, Tokyo 158-8501, Japan

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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[1] To whom correspondence should be addressed. Tel.: 81-3-3700-9064; Fax: 81-3-3707-6950; E-mail: yamaguch@nihs.go.jp.

[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 $\sim 10^6$ 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

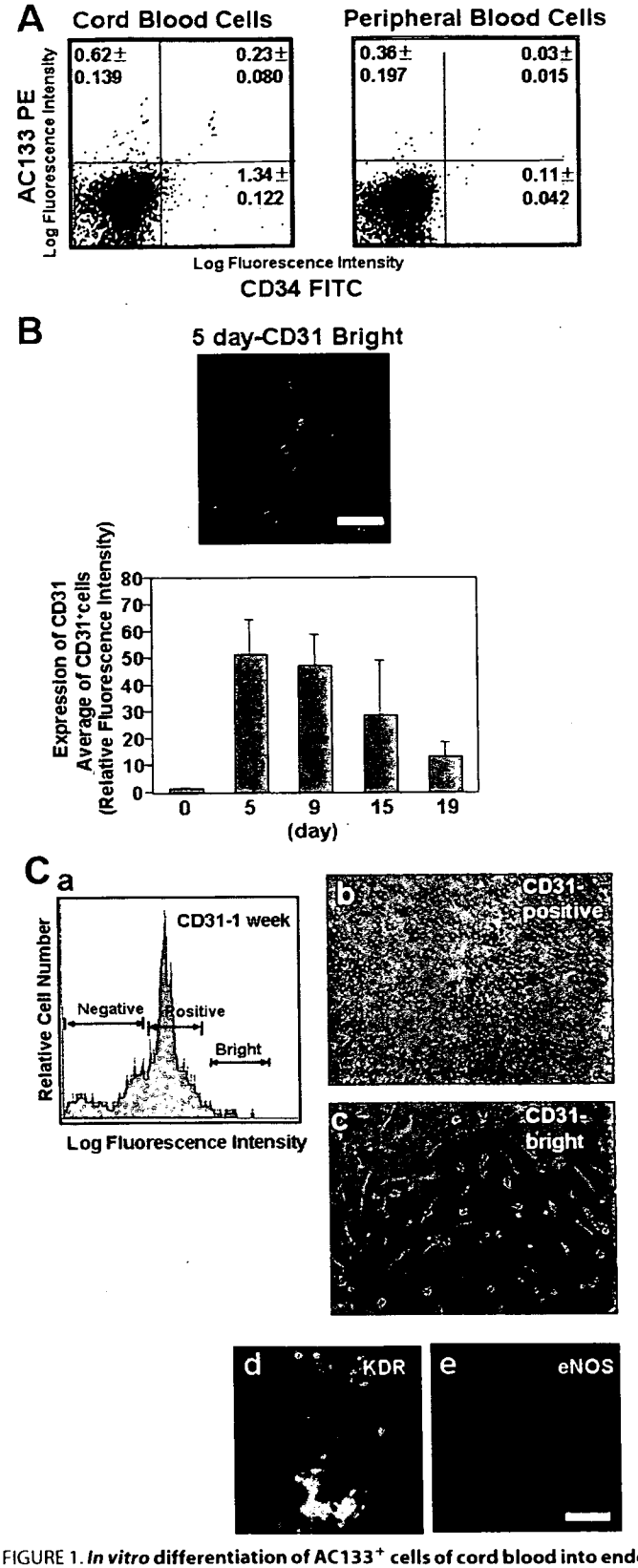

FIGURE 1. ***In vitro* differentiation of AC133$^+$ cells of cord blood into endothelial cells.** *A*, expression of AC133 and CD34 cells in human cord blood and peripheral blood mononuclear cells was analyzed by staining with AC133-PE (*vertical axis*) and CD34-FITC (*horizontal axis*). The *numbers* 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.

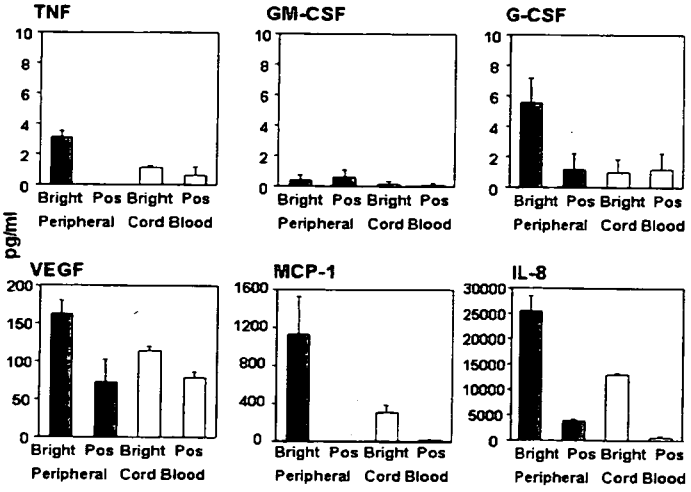

FIGURE 2. **Various cytokines released from CD31$^+$ cells and CD31^{bright} cells.** Production of various cytokines from CD31$^+$ cells and CD31^{bright} cells derived from AC133$^+$ cells cultivated for 5 days was measured. *Gray columns* indicate the cytokine production by cells from peripheral blood 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.

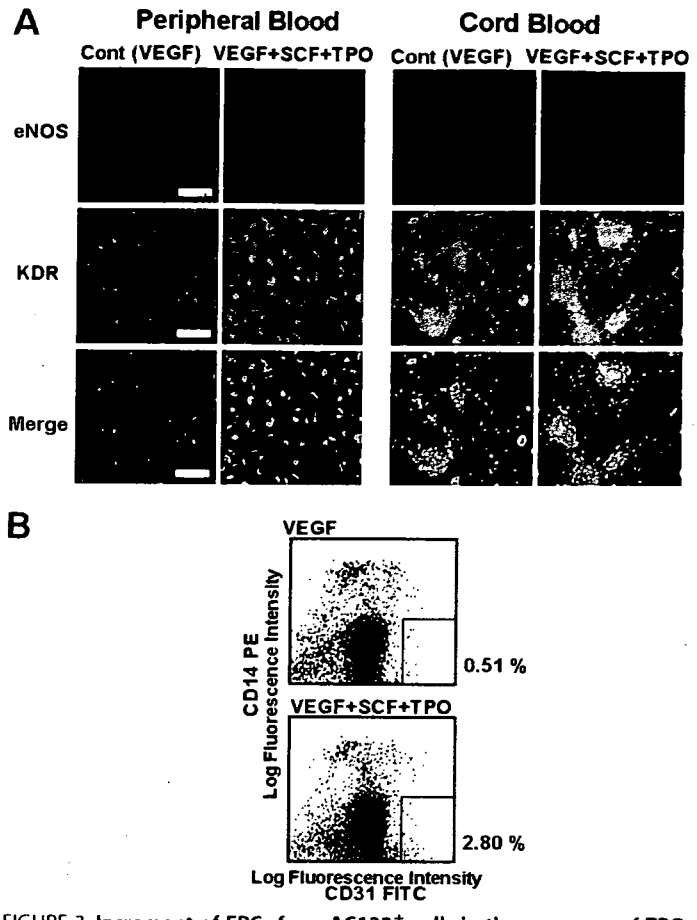

FIGURE 3. **Increment of EPCs from AC133$^+$ cells in the presence of TPO and SCF.** *A*, AC133$^+$ cells were differentiated for 2 weeks in the presence of either VEGF alone or the combination of TPO, SCF, and VEGF on an FN-coated dish. The *upper* and *middle panels* indicate the fluorescent photomicrographs 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

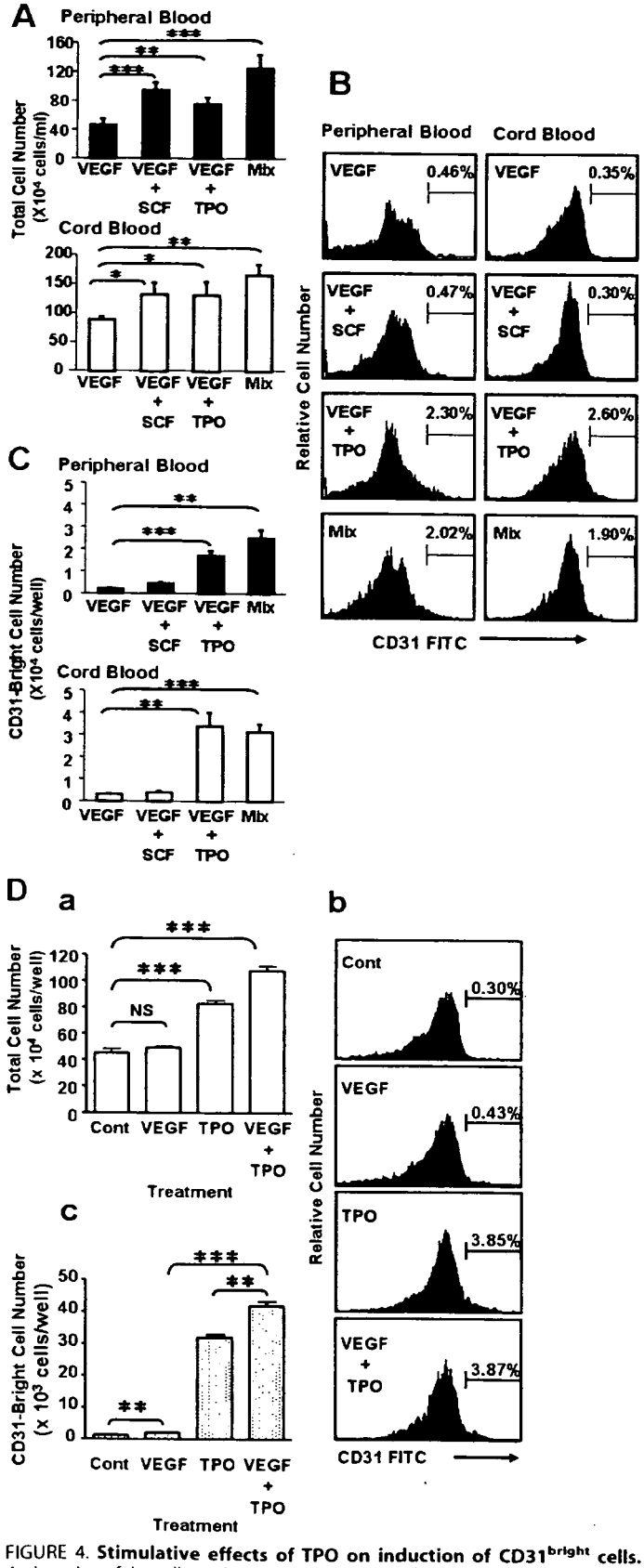

FIGURE 4. **Stimulative effects of TPO on induction of CD31^{bright} cells.** *A*, alteration of the cell number of cultivated AC133$^+$ cells for 1 week in the combination of growth factors. *Mix*, VEGF + SCF + TPO. *B*, the flow cytometric histogram of AC133$^+$-derived cells stained with FITC-labeled 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.

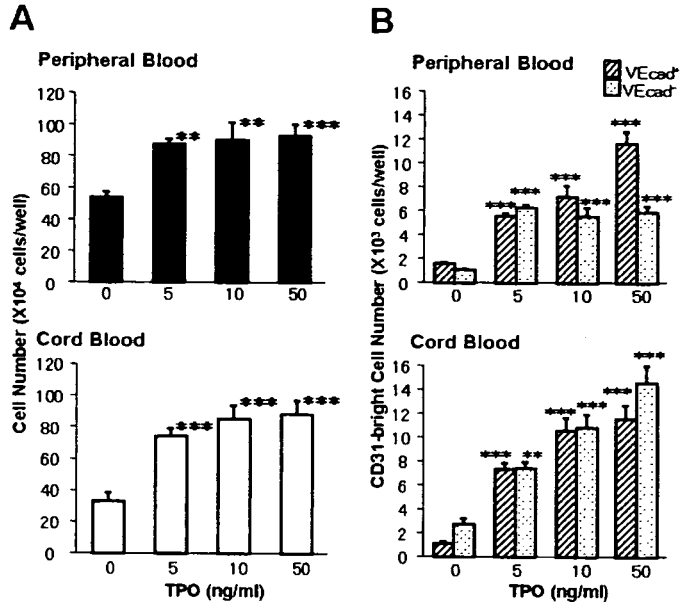

FIGURE 5. **Dose-dependent effects of TPO on the induction of CD31^{bright} cells from AC133$^+$ cells.** AC133$^+$ cells were treated with various concentrations of TPO for 1 week. The *left panels* (*A*) are the total cell number of cultured AC133$^+$ cells from peripheral blood (*upper panel*) and 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-

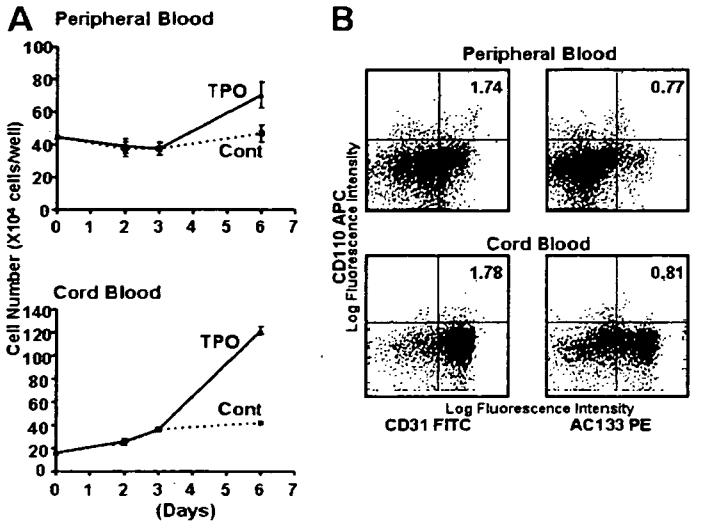

FIGURE 6. **Time-course analysis of TPO-treated AC133$^+$ cells and expression of TPO receptor (CD110).** *A*, alteration of cell number was counted at 2, 3, and 6 days. *Solid* and *dotted lines* indicate TPO-treated cells and control ((*Cont*) VEGF alone) cells, respectively. The results represent 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.

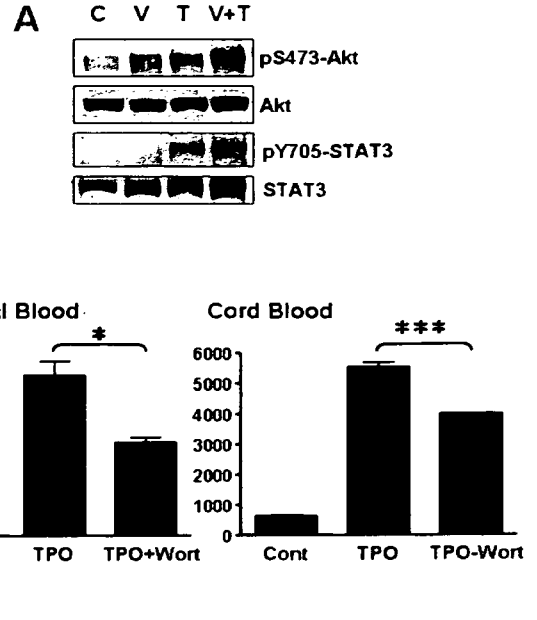

FIGURE 7. **Analysis of TPO-induced signal transduction on AC133$^+$ cells of cord blood.** *A*, activation of Akt or STAT3 was analyzed by Western blotting with anti-phospho-specific Ser-473-Akt antibody (*top panel*) and reprobed with anti-Akt antibody (*second panel*), or with anti-phospho-specific 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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