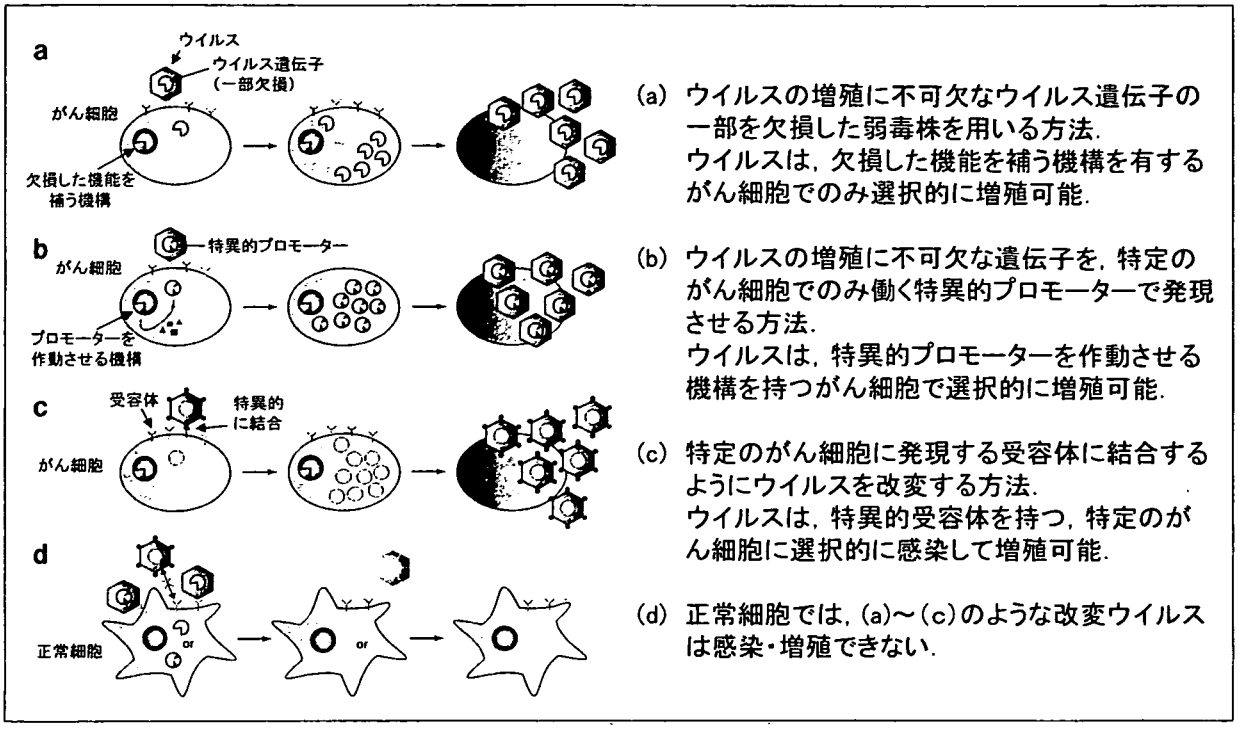

図2 ウイルスをがん細胞で選択的に増殖させる方法
(Ries SJ et al., 2004[13]. Fig 1 より改変)

果が得られるかの評価に，動物モデルが有用であることが一致した意見であった．しかし，① 腫瘍溶解性ウイルスの感染および複製能に動物種特異性があること，② 動物にヒト腫瘍細胞を移植した腫瘍モデル動物では，ウイルスがヒト体内とは異なる指向性・分布を示すこと，③ 動物での免疫反応がヒトとは異なること，などから動物モデルの限界も指摘されている．

しかし，生体内分布や安全性・毒性の評価，臨床での投与経路や用法・用量の選択などに関して，動物モデルが有用な情報を与えうるということについてはコンセンサスが得られている．腫瘍選択性に関しては，非腫瘍細胞培養株および腫瘍細胞培養株を用いた試験，またはヒト正常組織およびヒト腫瘍組織からの初代組織片培養を用いた試験が有用とされている．

（3） 臨床研究

腫瘍溶解性ウイルスは，その複雑な特性から，開発の基礎段階で充分に特性解析することが困難であり，また有用な動物モデルが必ずしも存在するわけではないことから，臨床研究の開始に当たっては多くの検討すべき課題があるとされた．

（4） 臨床薬物動態

臨床薬物動態の解析手法として，被験者のモニタリングにはPCR，感染性力価試験のいずれも用いられている．いくつかの腫瘍溶解性ウイルスの臨床研究において，血液中に検出されるウイルス量は投与直後と4～7日目にピークが認められた．このような2相性のピークは，局所投与および静注した場合のいずれでも観察されており，ウイルスの複製をモニターする手段となりうるとされている．

また，用法・用量設定の必要性，腫瘍溶解性ウイルスに対する患者の中和抗体の影響が重要な課題であるとされている．さらに，腫瘍溶解性ウイルスの体外排出に関する予防措置も大きな課題である．

（5） 腫瘍溶解性ウイルス開発の今後の展望

腫瘍溶解性ウイルスの開発の新たな流れとして，化学療法や放射線療法と腫瘍溶解性ウイルス療法を組み合わせる併用療法の有用性が示唆されており，今後このような併用療法の開発が進むものと考えられる．腫瘍溶解性ウイルスの設計改良のアプローチとしては，免疫反応を活性化する遺伝子などの治療

表5　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

1. 緒言
2. 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におけるリスク要因
   2.1　ベクター
   2.2　投与量および投与経路
3. 非臨床試験
   3.1　一般に考慮すべき事項
   3.2　生体内分布試験
4. 患者のモニタリング

用遺伝子をウイルスゲノムに挿入する遺伝子治療との組み合わせや，腫瘍細胞へのターゲティング能の増強などが行われている．また，殺腫瘍効果の作用機序を解明できるデータを得るための非臨床試験および臨床試験の取り組みも行われている．

現在，GT-DGでは，2008年を目途に，腫瘍溶解性ウイルスの品質・安全性評価に関するICH見解案を作成中である．

7. 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考え方

GT-DGでは，2005年から「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考え方」に関するICH見解をまとめるための議論が開始された．本見解の最も大きな目的は，遺伝子改変された次世代をつくらないということに尽きている．遺伝子治療用ベクターが生殖細胞の染色体へ組み込まれなくとも，生殖細胞へ遺伝子導入されただけでも発生毒性など他の安全性の懸念はもちろん生じるが，発生毒性などは他のガイドラインなどで対応していくものとされた．

本見解では，非臨床試験を通じて体内分布試験の実施によって生殖組織へのベクターの分布が起こるか否か，またベクターが生殖組織で検出された場合にはその検出が持続的か一過性かを明らかにすることを求めている．さらに，生殖組織で持続的に検出された場合，生殖細胞そのものに遺伝子が組み込まれているのか，白血球など周辺細胞にのみ局在するのかを明らかにすることが求められている．これらの試験を通じて，ベクターが生殖細胞内に持続的に保持されることが明らかになった場合には，ヒトへの使用に際しては規制当局と充分な議論をすることを求めている．

非臨床試験でベクターが一過性にせよ生殖腺に局在する可能性が示された場合には，臨床試験において患者の精液にベクターが局在することがないかモニタリングを考慮すべきとされた．また，臨床試験の期間中は，非臨床生体内分布試験の結果にかかわらず避妊手段をとるべきことが推奨された．なお，対象患者が生殖不能な場合，または余命が短いことが見込まれる重篤な疾患では，精液のモニタリングは必要ないとされている．

本見解は，2006年のICHシカゴ会議で最終案がとりまとめられ，ICH運営委員会によって承認された(表5)[17]．

GT-DGの今後の活動

ICH GT-DGの活動では，遺伝子治療をめぐって取り組むべき課題がより明確になりつつあることや，ここにきてICH各極で遺伝子治療用医薬品の規制当局への承認申請が出されていることへの早急な対応もあり，ICH見解の作成やガイドライン策定を見据えた議論も行われるようになってきている．今後，ベクターの排出に関する見解の作成やガイドライン化，腫瘍溶解性ウイルスに関する見解の作成など，いくつかの重要な科学的コンセプトが出されていく予定になっている．

わが国における遺伝子治療の臨床研究の数は欧米にくらべて非常に少なく，臨床研究での情報は多くが海外に依存している現況であることは否めない．しかし，ここ数年はわが国においても遺伝子治療薬の開発が急速に進んでおり，遺伝子治療薬に関するICH見解やガイドラインの策定が，わが国における遺伝子治療薬開発の促進につながっていくと期待される．

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Study on the quality control of cell therapy products Determination of *N*-glycolylneuraminic acid incorporated into human cells by nano-flow liquid chromatography/Fourier transformation ion cyclotron mass spectrometry

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Abstract

N-Glycolylneuraminic acid (NeuGc), an acidic nine-carbon sugar, is produced in several animals, such as cattle and mice. Since human cells cannot synthesize NeuGc, it is considered to be immunogenic in humans. Recently, NeuGc contamination was reported in human embryonic stem cells cultured with xenogeneic serum and cells, suggesting that possibly NeuGc may harm the efficacy and safety of cell therapy products. Sialic acids have been determined by derivatization with 1,2-diamino-4,5-methylenedioxybenzene (DMB) followed by liquid chromatography/mass spectrometry (LC/MS) and liquid chromatography/tandem mass spectrometry (LC/MS/MS); however, the limited availability of cell therapy products requires more sensitive and specific methods for the quality test. Here we studied the use of nano-flow liquid chromatography/Fourier transformation ion cyclotron resonance mass spectrometry (nanoLC/FTMS) and nanoLC/MS/MS for NeuGc-specific determination at a low femtomole level. Using our method, we found NeuGc contamination of the human cell line (HL-60RG cells) cultured with human serum. Our method needs only 2.5×10^3 cells for one injection and would be applicable to the determination of NeuGc in cell therapy products.

Keywords: *N*-Glycolylneuraminic acid; Nano-flow liquid chromatography; Fourier transformation ion cyclotron mass spectrometry; Cell therapy products

1. Introduction

Sialic acids are a family of acidic nine-carbon sugars found in the non-reducing terminal of *N*-linked and *O*-linked oligosaccharides of glycoproteins and glycolipids [1,2]. There are more than 30 members with different substitutions on the amino group at carbon 5 and on hydroxyl groups at carbons 4, 7, 8 and 9 [2–8]. *N*-Glycolylneuraminic acid (NeuGc), a 5-*N*-glycolylated sialic acid, is produced in several animals, such as cattle, horses, mice and rats [9]. Since human cells cannot synthesize NeuGc due to mutation of the cytidine monophospho (CMP)-*N*-acetylneuraminic acid (NeuAc) hydroxylase gene [10,11], NeuGc is considered to be antigenic and to induce immunoreaction in humans [4,12,13].

Advances in biotechnology and cell culture techniques make it possible to administer human and animal cells directly to patients as cell therapy products. In cell therapy and tissue engineering, human embryonic stem (ES) cells are expected to be useful for the treatment of many diseases. Recently, it was reported that NeuGc is incorporated into ES cells from human and mouse feeder cells and cultivation media containing xenogeneic serum, such as fetal calf serum (FCS) [14,15]. Since NeuGc is a foreign component in humans, it is feared that NeuGc may harm the efficacy and safety of cell therapy products. To

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assess the adverse effects of NeuGc, it is necessary to quantify NeuGc in cell therapy products.

Sialic acids have been determined by labeling with 1,2-diamino-4,5-methylenedioxybenzene (DMB) followed by conventional high-performance liquid chromatography (HPLC) with fluorescent detection [16–20]. The femtomole level of sialic acid can be determined by fluorescent detection [19]. The use of liquid chromatography/mass spectrometry (LC/MS) and liquid chromatography/tandem mass spectrometry (LC/MS/MS) has more advantage in the identification of sialic acid species [18,20–22]. The derivatization of sialic acids with DMB has advantages of good separation of NeuGc from NeuAc in chromatography and enhancement of ionization efficiency in MS. However, more sensitive and specific methods are desired for the quality control of cell therapy products, since in many case only a low number of cell products, approximately 1×10^6 to 1×10^8, should be available for quality tests.

In this study, we studied the use of nano-flow liquid chromatography/Fourier transformation ion cyclotron resonance mass spectrometry (nanoLC/FTMS) and LC/MS/MS to achieve the sensitive and specific determination of NeuGc. The potential of the method for quality testing of cell therapy products was evaluated using substrain of human promyelocytic leukemia HL-60 cells (HL-60RG cells) as model cells. Using this method, we determined NeuGc in membrane fractions from HL-60RG cells cultured with FCS, human serum and serum-free medium.

2. Experimental

2.1. Materials

NeuGc and NeuAc were purchased from Nacalai Tesque (Kyoto, Japan). FCS and normal human serum were purchased from Dainippon Sumitomo Pharma (Osaka, Japan). RPMI1640 medium and ASF104 medium were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Ajinomoto (Tokyo, Japan), respectively.

2.2. Cell culture

Substrain of human promyelocytic leukemia HL-60 cells (HL-60RG cells, JCRB Cellbank, Osaka, Japan) was cultured in RPMI1640 medium supplemented with 10% FCS, 100 unit/ml of penicillin and 100 μg/ml of streptomycin under a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. HL-60RG cells were replaced at 2×10^5 cells/100 mm dish in RPMI1640 medium supplemented with 10% FCS or 10% normal human serum, and in serum-free ASF104 medium. The media were replaced four times, and semi-confluent growth cells were harvested.

2.3. Fractionation of the membrane fraction

The cells were washed in phosphate buffer saline (PBS) supplemented with protease inhibitors (protease inhibitor mix solution, Wako, Tokyo, Japan) three times. The washed cells (1×10^6) were suspended in 100 μl of 0.25 M sucrose/10 mM Tris–HCl buffer (pH 7.4) containing protease inhibitors, and sonicated at 4 °C for 30 s, two times (40W, Bioruptor UCW-201, Tosyoudenki, Kanagawa, Japan). After the nuclei were removed by centrifugation at 4 °C, $450 \times g$ for 10 min, the mitochondria and lysosome fractions were removed by re-centrifugation at 4 °C, $20{,}000 \times g$ for 10 min. The membrane fractions were precipitated by ultracentrifugation at 4 °C, $100{,}000 \times g$ for 60 min. The membrane fractions were washed in 100 μl of 150 mM ammonium acetate buffer (pH 7.4) and recovered by re-ultracentrifugation.

2.4. Derivatization of NeuGc and NeuAc with DMB reagent

The membrane fractions were sonicated in 250 μl of H_2O and then incubated with 250 μl of 4 M acetic acid (final concentration, 2 M) at 80 °C for 3 h. The released sialic acids were passed though a solid-phase extraction cartridge (SepPak C-18, Waters, Milford, MA, USA) with 2 ml of H_2O, dried under vacuum, and resolved in 50 μl of H_2O. The solution was incubated with DMB according to the manufacturer's instruction (Takara, Tokyo, Japan), and the reaction mixture was applied on a solid-phase extraction cartridge (Envi-Carb C, Supelco, Bellefonte, PA, USA). After washing the cartridge with 2.5 ml of 5 mM ammonium acetate (pH 9.6) for desalting, the DMB-labeled sialic acids were eluted with 3 ml of 45% acetonitrile/5 mM ammonium acetate (pH 9.6). The collected fraction was freeze dried.

2.5. nanoLC/FTMS

DMB-labeled sialic acids were separated by HPLC using Paradigm MS4 (Michrom BioResource, Auburn, CA, USA) equipped with a reversed-phase C18 column (Magic C18, 50 mm × 0.1 mm, 3 μm, Michrom BioResource, Auburn, CA, USA). Elution was achieved using 0.1% formic acid/2% ace-

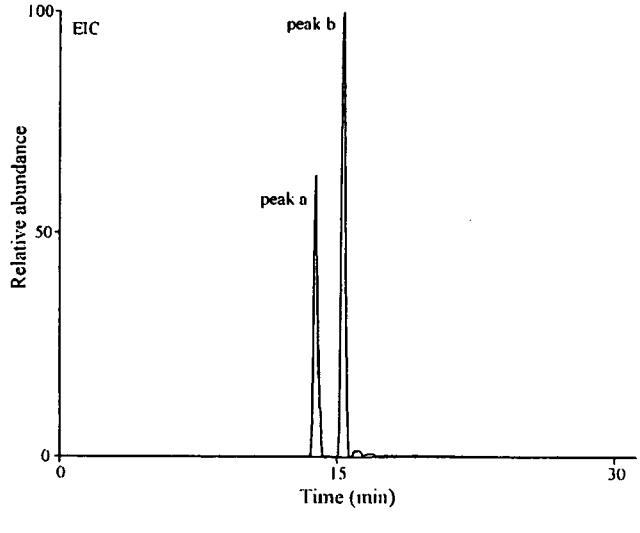

Fig. 1. EIC at *m/z* 426.13–426.17 and *m/z* 442.12–442.16 obtained by SIM (*m/z* 400–450) of DMB-NeuGc and DMB-NeuAc in the positive ion mode.

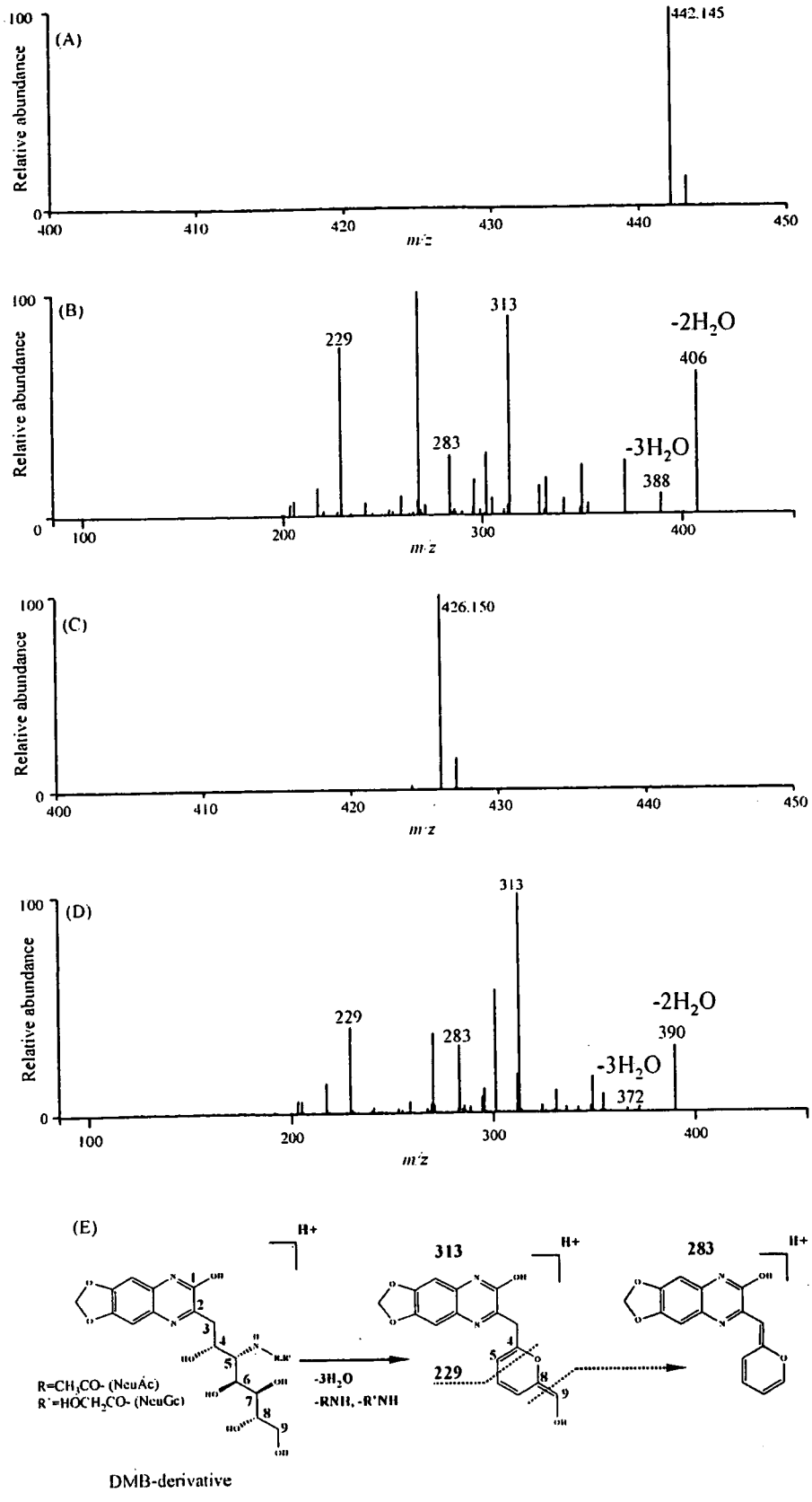

Fig. 2. (A) Typical MS spectrum of peak a. (B) MS/MS spectrum of $[M+H]^+$ (*m/z* 442.145) acquired from around peak a. (C) Typical MS spectrum of peak b. (D) MS/MS spectrum of $[M+H]^+$ (*m/z* 426.150) acquired from around peak b. (E) Fragmentation of DMB-NeuGc and DMB-NeuAc.

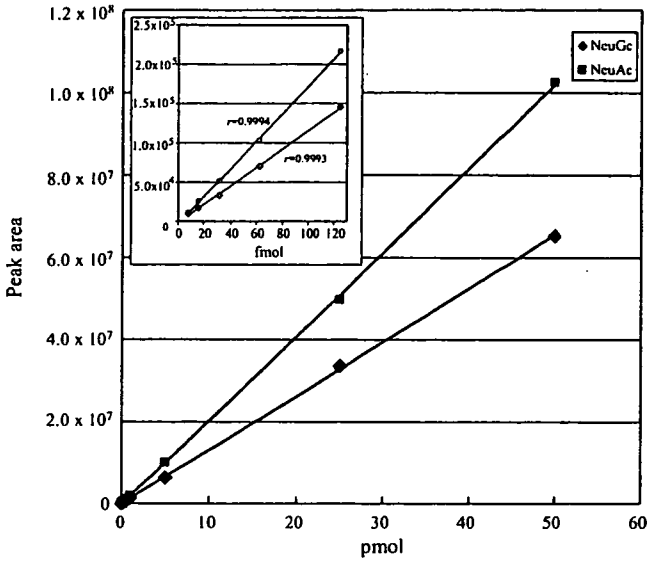

Fig. 3. Calibration curves of DMB-NeuGc ($r=0.9998$) and DMB-NeuAc ($r=0.9995$).

tonitrile (pump A) and 0.1% formic acid/80% acetonitrile (pump B) with a linear gradient of 10–90% of B in 30 min at a flow rate of 750 nl/min. On-line MS and MS/MS were performed using an Fourier transformation ion cyclotron resonance (FT)/ion trap (IT) type mass spectrometer (LTQ-FT, Thermo-Electron, San Jose, CA, USA) equipped with a nanoelectrospray ion source (AMR, Tokyo, Japan). DMB-NeuAc and DMB-NeuGc were determined by selected ion monitoring (SIM) in the positive ion mode. The analytical conditions were set to 200 °C for capillary temperature, 1800 eV spray voltage, *m/z* 400–450 scan range, and 35% collision energy. The automatic gain control (AGC) value, which is adjusted for the amount of imported ions for FTMS, was set to 5×10^4. Maximum injection times, which are the adjusted times of imported ions, for ITMS and FTMS, were set to 50 and 1250 ms, respectively.

2.6. Method validation

The linearity of the signal intensity peak area of DMB-NeuAc and DMB-NeuGc was assessed by injections of 0.0078–500 pmol DMB derivatives. Correlation coefficients were calibrated using a least-squares linear regression model. The detection limit (DL) and the quantification limit (QL) were calculated using the formulas DL = 3.3 × σ/slope (σ: average of noise on chromatograph) and QL = 10 × σ/slope, respectively. Accuracy and precision were determined by measuring three samples, where NeuGc spiked at the concentration of 50 fmol to the membrane fraction of cells cultured in serum-free medium which contains no NeuGc before the derivatization of NeuGc with DMB. Accuracy was calculated by comparison of the mean peak area and the calibration curve. Precision was estimated by relative standard deviation (RSD) from three samples.

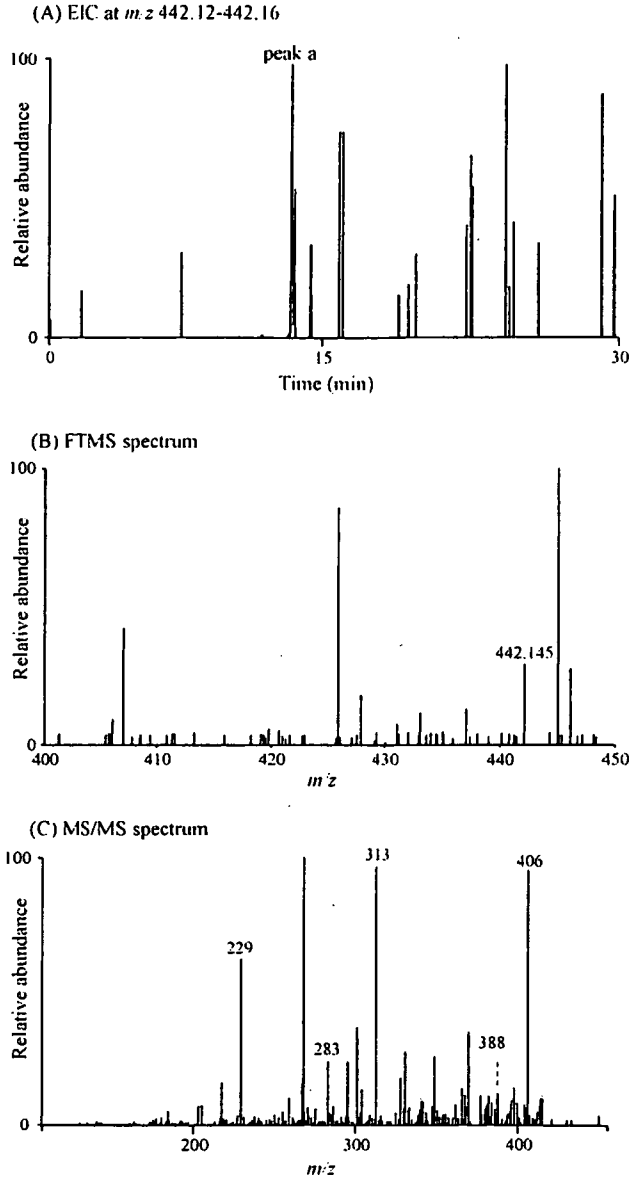

Fig. 4. Detection of DMB-NeuGc in the membrane fractions of HL-60RG cells (2.5×10^3) cultured with 10% FCS. (A) EIC at *m/z* 442.12–442.16 obtained by SIM. (B) Typical MS spectrum of peak a. (C) MS/MS spectrum of $[M+H]^+$ (*m/z* 442.145) acquired from around peak a.

3. Results and discussion

3.1. Analysis of NeuGc and NeuAc by nanoLC/FTMS

It was reported that DMB-NeuGc yielded its dehydrated ion (*m/z* 424) together with molecular ion (*m/z* 442) by MS in the positive ion mode [18,21]. To control the dehydration of molecular ion in the ion trap device, AGC value, which regulates the amount of ions trapped into ion trap device, was set to 5×10^4 (default value, 5×10^5). This value was also useful for the detection of molecular ion of DMB-NeuAc.

Using the AGC value at 5×10^4, SIM (*m/z* 400–450) was carried out in the positive ion mode. When a mix-

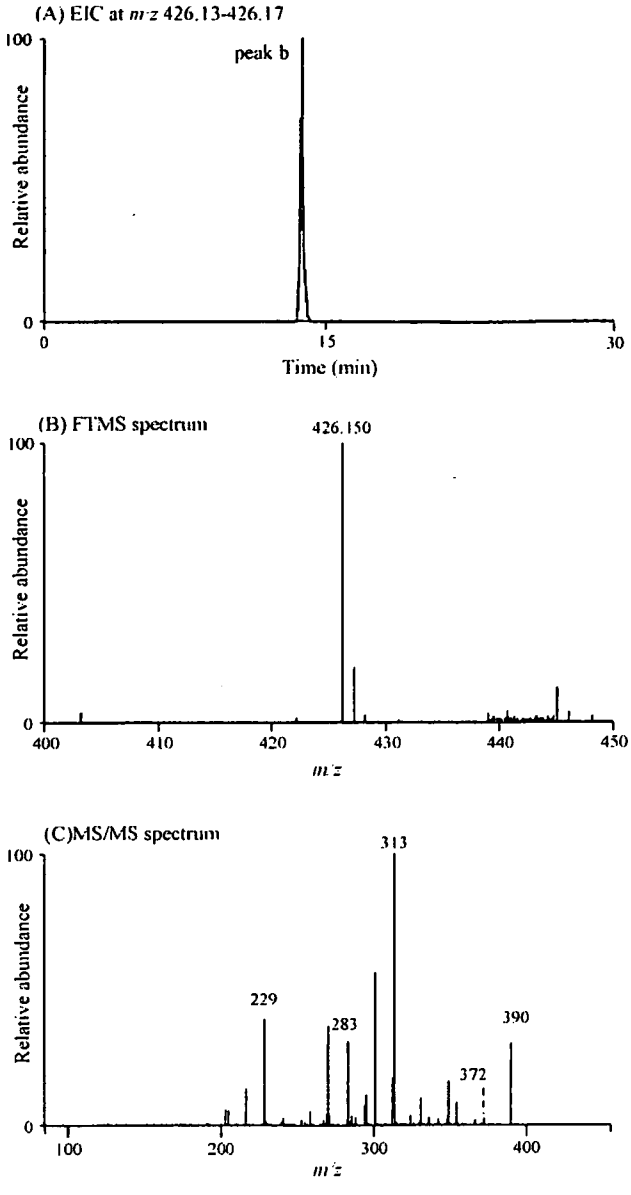

Fig. 5. Detection of DMB-NeuAc in the membrane fractions of HL-60RG cells (2.5×10^3) cultured with 10% FCS. (A) EIC at *m/z* 426.13–426.17 obtained by SIM. (B) Typical MS spectrum of peak b, (C) MS/MS spectrum of $[M+H]^+$ (*m/z* 426.150) acquired from around peak b.

ture of DMB-NeuGc and DMB-NeuAc (2 pmol each) was subjected to nanoLC/MS, two peaks appeared at 14 min (peak a) and 15 min (peak b) on the extracted ion chromatogram (EIC) at *m/z* 426.13–426.17 and *m/z* 442.12–442.16 (Fig. 1).

As shown in Fig. 2A, the *m/z* values of molecular ions around 14 min (*m/z* 442.145) suggest the elution of DMB-NeuGc in peak a. The structure of the DMB derivative at peak a was confirmed by the product ion spectra acquired from $[M+H]^+$ (*m/z* 442.145) as a precursor ion (Fig. 2B). Product ions missing two and three molecules of H_2O were found at *m/z* 406 and 388 in MS/MS spectra. Ions losing three H_2O and glycolyl groups (*m/z* 313), cross-ring fragment ion (*m/z* 229) and fragment ion yielded by loss of formaldehyde (*m/z* 283) were also formed by

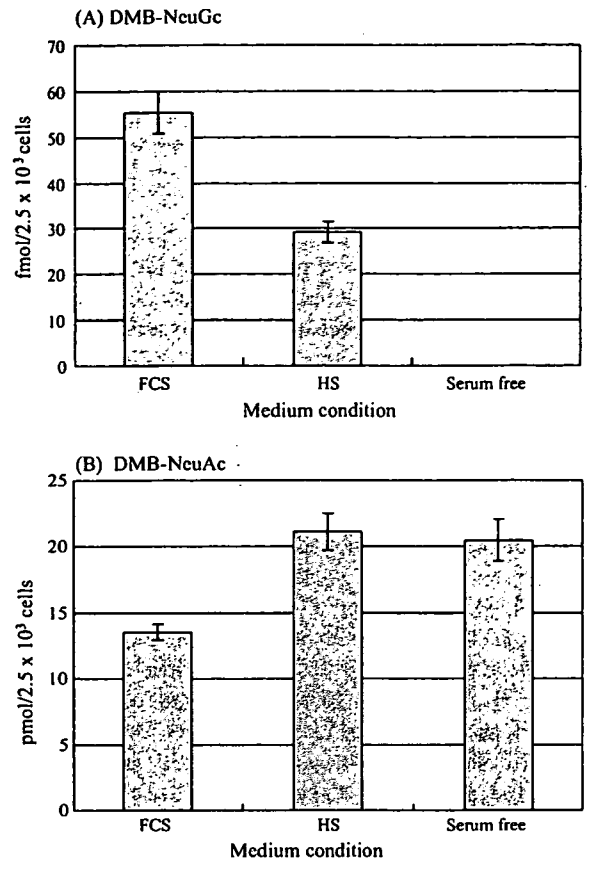

Fig. 6. Levels of (A) NeuGc and (B) NeuAc in the membrane fraction of HL-60RG cells (2.5×10^3) cultured with 10% FCS, 10% human serum (HS) and serum-free medium. Values are the means ± SD ($n = 3$).

MS/MS (Fig. 2E). The fragment pattern of the MS/MS spectrum from $[M+H]^+$ (*m/z* 442.145) was consistent with that of DMB-NeuGc in the previous report [21]. Fragments at *m/z* 406 and 388 are DMB-NeuGc characteristic ions, which could be used for specific determination of DMB-NeuGc. Likewise, peak b was identified as DMB-NeuAc by molecular ions (*m/z* 426.150) and their product ions (*m/z* 390, 372, 313, 283 and 229) formed by MS/MS of $[M+H]^+$ (*m/z* 426.150) as a precursor ion (Fig. 2C and D).

Calibration curves were prepared by the injection of DMB-NeuGc and DMB-NeuAc from 0.0078 to 500 pmol. The linearity of DMB-NeuGc and DMB-NeuAc was confirmed in the range of 0.0078–50 pmol with the regression equations of $Y = 1.31 \times 10^6 X - 9028.5$ ($r = 0.9998$) and $Y = 2.03 \times 10^6 X - 21548.0$ ($r = 0.9995$), respectively (Fig. 3). DL and QL of DMB-NeuGc were 8.6 and 26.3 fmol, and those of DMB-NeuAc were 5.6 and 16.9 fmol, respectively. The use of FT/MS gave an accuracy of 92.4% by eliminating contaminants by using accurate *m/z* values. The precision of this method for NeuGc was 7.3%. Compared to the former method, in which a micro or semi-micro column and the quadrupole mass spectrometer were used for the detection of picomole levels of DMB derivatives, SIM by using nanoLC/FTMS achieved the specific detection of DMB-derivatized sialic acids at a lower level. The method using nanoLC/FTMS and nanoLC/MS/MS allows not

only the determination of DMB-derivatives with similar sensitivity as the fluorescence detection but also the identification of sialic acid species.

3.2. Quantification of NeuAc and NeuGc in membrane fraction of HL-60RG cells

Using HL-60RG cells as model cells, the potential of this method for the quantification of NeuGc on the cell membrane was evaluated. The membrane fraction from cells (1×10^6) cultured with 10% FCS was prepared by ultracentrifugation. Sialic acids were released by treatment with 2 M acetic acid at 80 °C for 3 h and derivatized with DMB. DMB derivatives (2.5×10^3 cells) were subjected to nanoLC/MS and nanoLC/MS/MS in SIM mode. As shown in Fig. 4A, some peaks appeared in EIC at *m/z* 442.12–442.16. Based on the retention time as well as the *m/z* value of molecular ion (*m/z* 442.145), peak a that appeared at 14 min was assigned to be a peak of NeuGc (Fig. 4B). Fig. 4C shows the MS/MS spectrum acquired from $[M+H]^+$ (*m/z* 442.145) as precursor. The NeuGc-characteristic ions at *m/z* 406 and 388 together with other product ions at *m/z* 313, 283 and 229 clearly indicate the presence of NeuGc in the membrane fraction of HL-60RG cells. In the EIC at *m/z* 426.13–426.17, the single peak was observed at 15 min (Fig. 5A). The molecular ion at *m/z* 426.150, and product ions at *m/z* 390, 372, 313, 283 and 229 acquired at 15.13 min suggest that DMB-NeuAc is eluted in peak b (Fig. 5B and C). The levels of NeuGc and NeuAc in the membrane fraction from HL-60RG cells (2.5×10^3 cells) cultured with 10% FCS were 55.4 ± 4.6 fmol and 13.5 ± 0.6 pmol, respectively (Fig. 6)

After the cultivation of HL-60RG cells with human serum for 10 days (medium was changed four times), NeuGc and NeuAc were determined by the proposed method. Fig. 7A shows the EIC at *m/z* 442.12–442.16 obtained by nanoLC/MS. In spite of cultivation in human serum, an obvious peak still appeared at 14 min. Molecular ion (*m/z* 442.145) and NeuGc-characteristic product ions found in the MS/MS spectrum acquired from the molecular ion clearly indicate the presence of NeuGc in the membrane fraction (Fig. 7B and C). The levels of NeuGc and NeuAc in cells (2.5×10^3) cultured in 10% human serum were 29.2 ± 2.4 fmol and 21.0 ± 1.4 pmol, respectively (Fig. 6).

In contrast, no significant peaks appeared in EIC at *m/z* 442.12–442.16 when HL-60RG cells were cultured in serum-free medium for 14 days (medium was changed four times). The level of NeuAc in cells cultured in serum-free medium was 20.5 ± 1.6 pmol (Fig. 6).

As shown in Figs. 4A and 7A, there are many different molecules detected at *m/z* 442.14–442.16 in the cells, which makes it difficult to determine a small amount of NeuGc in the membrane fraction by the low-resolution mass spectrometry. The DMB-NeuGc-specific detection was achieved by acquisition of both the accurate mass by FTMS and the characteristic product ions arisen from DMB-NeuGc by MS/MS.

Our method needs only 2.5×10^3 cells for one injection and is applicable to the determination of NeuGc in cell therapy products. The incorporation of dietary NeuGc into human serum has been reported by Tangvoranuntalul et al. [23], which has raised concerns about NeuGc contamination of cell therapy products through cultivation with human serum. Although using our method, we demonstrated the existence of NeuGc in human cells cultured with human serum, NeuGc could not be detected in human cells cultured in serum-free medium in which no NeuGc exists. These results suggest the difficulty of avoiding NeuGc contamination of cell therapy products during the manufacturing process. Further study to assess the immunogenicity of incorporated NeuGc is necessary to ensure the safety and efficacy of cell therapy products, and our method is useful for the sensitive and quantitative analysis of NeuGc in cell therapy products.

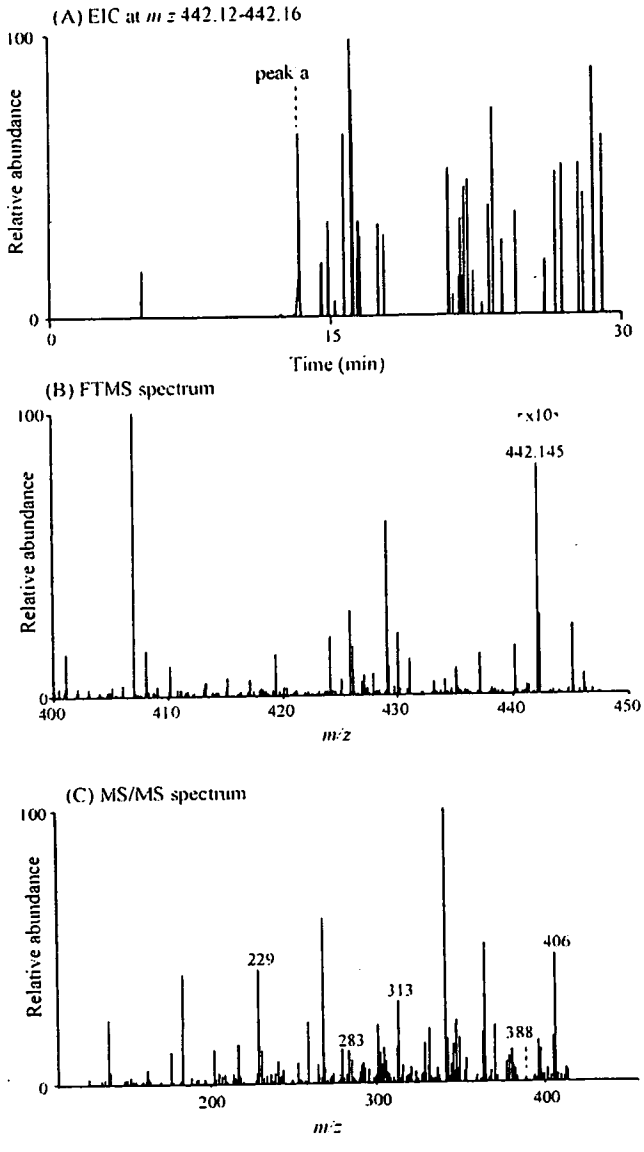

Fig. 7. Detection of DMB-NeuGc in the membrane fractions of HL-60RG cells (2.5×10^3) cultured with 10% human serum. (A) EIC at *m/z* 442.12–442.16 obtained by SIM. (B) Typical MS spectrum of peak a. (C) MS/MS spectrum of $[M+H]^+$ (*m/z* 442.145) acquired from around peak a.

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Regulatory Aspects of Oncolytic Virus Products

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Abstract: Many types of oncolytic viruses, wild-type virus, attenuated viruses and genetically-modified viruses, have been developed as an innovative cancer therapy. The strategies, nature, and technologies of oncolytic virus products are different from the conventional gene therapy products or cancer therapy products. From the regulatory aspects to ensure the safety, efficacy and quality of oncolytic viruses, there are several major points during the development, manufacturing, characterization, non-clinical study and clinical study of oncolytic viruses. The major issues include 1) virus design (wild-type, attenuated, and genetically engineered strains), 2) poof of concept in development of oncolytic virus products, 3) selectivity of oncolytic virus replication and targeting to cancer cells, 4) relevant animal models in non-clinical studies, 5) clinical safety, 6) evaluation of virus shedding. Until now, the accumulation of the information about oncolytic viruses is not enough, it may require the unique approach to ensure the safety and the development of new technology to characterize oncolytic viruses.

Keywords: Gene therapy, cancer therapy, replicating virus.

INTRODUCTION

Oncolytic virus therapy has been developed as a new wave of cancer therapies. These therapies are dependent on the replication-selective nature of these viruses in tumor cells, which provides the marked breaths of cancer therapy. More than one century ago, evidence of oncolytic activity caused by replicating viruses was reported, and it was known the viruses could induce tumor lysis. Using these studies as a point of departure, rare but dramatic responses in cancer patients recovering from viral infections were reported. In the early development of oncolytic virus therapy, wild-type viruses with low pathogenicity to normal tissues, or attenuated viruses were selected for the treatment of cancer patients. However, some adverse events, such as the development of encephalitis in immune compromised patients, were reported [1-3]. Other works reported the oncolytic activity of wild-type or attenuated oncolytic viruses to be transient or limited to the site of injection [4-8]. Recently, attention has focused on overcoming the disadvantages of wild-type or attenuated oncolytic virus therapy, and many genetically modified viruses have been developed for cancer treatment. Progress in the development of genetically engineered oncolytic viruses has been based on recent advances in our understanding of the molecular biology of cancer and viruses, and on advances in genetic engineering technologies of virus genomes. Although many gene therapy clinical studies for the treatment of cancer have been conducted during the past decade using replication-incompetent virus vectors, these studies have not achieved satisfying results. Tumor-selective replicating viruses have been suggested to have an advantage over conventional gene therapy vectors for cancer therapy, and oncolytic viruses, especially genetically modified viruses, must be considered to be a special type of gene therapy product since their principle is directly associated with the transfer of the viral genome as the therapeutic gene [9]. In the present report, we review the development of oncolytic viruses as gene therapy products or attenuated virus products with specific reference to the associated regulatory issues.

Oncolytic virus therapy is based on several strategies, including tumor-selective replication, tumor-selective targeting, and/or the minimization of toxicity to normal cells. Many types of viruses have been utilized in oncolytic virus therapy; including adenovirus, herpes simplex virus (HSV), reovirus, Newcastle disease virus (NDV), vaccinia, measles virus, vesicular stomatitis virus (VSV) and Sendai virus [10-13]. During the development of oncolytic virus products, a number of major issues have arisen with respect to ensuring the quality, safety and efficacy of the products: 1) virus design (wild-type, attenuated and genetically engineered strains); 2) proof of concept in the development of oncolytic virus products; 3) the selectivity of oncolytic virus replication and targeting to cancer cells; 4) relevant animal models in non-clinical studies; 5) clinical safety; and 6) the evaluation of virus shedding. Since the strategies, nature and technologies of oncolytic virus products are different from those of conventional gene therapy products or cancer therapy products, we discuss the regulatory aspects of the development of oncolytic viruses in the present paper.

VIRUS DESIGN AND PRODUCT DEVELOPMENT

While many types of viruses are utilized for oncolytic virus therapies [10, 11, 14], selective replication in tumor cells is essential for the efficacy and safety of oncolytic viruses. Wild-type viruses and naturally occurring attenuated viruses are known to possess the ability to infect and kill transformed cells such as tumor cells. For example, VSV, NDV and reovirus have been used as oncolytic viruses with inherent tumor-selectivity [12, 15-18]. In the case of wild-type viruses or attenuated viruses, the mechanism underlying the virus-selectivity to tumor cells has been analyzed from various points of view. For instance, reovirus has an inherent preference for replicating cells with dysregulated growth factor-signaling cascades by Ras activation [17, 19]. Attenuated strains from HSV-1 have been reported to be potential anti-cancer therapeutics and have necessitated a thorough investigation into the molecular basis of host-cell permissiveness to HSV [20-22]. Since in the

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development of wild-type or naturally attenuated oncolytic viruses, intentional genetic modification is not introduced into the virus genome, the tumor selective targeting, tumor-selective replication and pathogenicity of these oncolytic viruses are dependent on the method of selecting the strains. During the characterization of wild-type and attenuated oncolytic viruses, it is extremely important to analyze the molecular mechanisms of the tumor-selectivity and tumor-specific replication, as well as the genetic stability, etc.

There are several strategies used to design and construct the tumor selectivity of genetically engineered oncolytic viruses [10, 11, 23, 24]. One strategy is to engineer viruses through the deletion of virus genes critical for viral replication in normal cells but non-essential in tumor cells. For example, adenovirus E1B55K is responsible for binding and inactivating p53. E1B55K-deleted adenovirus has acquired the ability to propagate and induce cell death selectively in tumor cells, and then to spread to surrounding cells and tissues [25, 26]. Another strategy is transcriptional targeting, in which tumor- or tissue-specific promoters that are active in tumor cells are inserted into the viral genome to regulate the expression of essential viral genes and to restrict viral replication in tumor cells. The promoters used are categorized into different types; telomerase reverse transcriptase (TERT), S-phase of cell cycle promoter (E2F) and hypoxia promoter (HIF-1) are utilized as targeting promoters to all tumor cells; and prostate-specific antigen (PSA) promoter, α-fetoprotein (hepatoma) promoter and albumin promoter for hepatoma are used as tissue-specific promoters. The third strategy is the receptor-mediated targeting of replication-competent viruses to tumor cells [23, 27]. In this approach, the natural virus tropism of a replication-competent virus is adapted to the tumor cells through genetic modification of the virus coat or envelopes. This approach requires the ablation of the natural virus infection pathway and the introduction of new ligands into the virus surface without disrupting viral integrity. To improve the selectivity of oncolytic viruses to tumor cells and to improve safety, constructs with multiple modifications to tumor selectivity are developed. In addition, to improve efficacy, oncolytic viruses carrying a transgene (armed oncolytic viruses) have recently been developed [14, 28, 29].

In the endorsement of clinical trials or the approval of oncolytic virus products, the scientific rational of the design of the oncolytic virus construct must be thoroughly justified. Furthermore, non-clinical studies should be designed in each case to verify predictions of efficacy and safety. In this context, it is recommended that animal models be developed to provide valuable evidence concerning the non-clinical safety of these products and to assess their proof of concept.

MANUFACTURING AND CHARACTERIZATION OF ONCOLYTIC VIRUSES

There are to date no specific regulatory guidelines related to the manufacture and characterization of oncolytic viruses for clinical use. However, guidelines concerning the manufacturing and characterization of gene therapy products have been issued by the Food and Drug Administration (FDA), the European Medicines Agency (EMEA) and the government of Japan [30-33]. While there are some differences in the format of these guidelines, the underlying scientific principles are not fundamentally different, and the scientific principles covered by the above guidelines for gene therapy products may be applicable to the evaluation of the manufacturing and characterization of oncolytic viruses.

The guidelines should require that the rational behind the selection of the virus, helper virus and cells used in the production of the virus be described, including the genetic construct of the oncolytic virus, and of the helper virus if applicable. In cases in which the manufacturing method of the oncolytic virus in question has a specific feature, a justification of the feature must be included. The DNA or RNA sequence of the oncolytic virus must be clarified as much as possible, with particular attention to any regions of the virus genome that have been modified. Sequence analysis should be performed by a validated method which must also be described. In the case of genetically modified oncolytic viruses, a full explanation must be provided of the origin and detailed derivation of all constitutive components, such as promoters, enhancers, duplication units, selection markers and other base sequence parts from other constructs of oncolytic virus DNA or RNA. When a transgene is inserted into an oncolytic virus sequence, the construing procedure, amplification method, purification method and any flanking area that may have an important effect on the transcription, translation or stability of the translation sequence must be described in detail.

Cell and Virus Bank System

It is important to establish a cell and virus banking system in order to maintain consistency in the production of oncolytic viruses. A cell banking system for manufacturing oncolytic viruses should be designed and fully characterized; in general, a cell banking system includes a Master Cell Bank (MCB) and Working Cell Bank (WCB) for producing and packaging cell lines ("International conference on harmonization of technical requirements for registration of pharmaceuticals for human use (ICH)" guideline Q5D [34]). The concept of a two-tiered cell bank, in which the MCB is used to generate WCBs, is generally considered to be the most practical approach to providing a supply of cell substrates for the continued manufacture of oncolytic virus products. The strategy for providing a continued supply of cells from their cell bank(s) must be described, including the anticipated utilization rate of the cell bank(s) for production, the expected intervals between the generations of new cell bank(s), and the criteria for qualification of cell bank(s). Generally, the MCB is created first, usually directly from an initial clone or from a preliminary cell bank derived from an initial clone. A WCB is derived from one or more containers of the MCB. It is the WCB which is typically used to directly provide cells for the manufacturing process.

The characterization and testing of banked cells is critical for the control of oncolytic viruses. The MCB and WCB must be subject to extensive quality control, and the established guidelines will be applicable to evaluate these banks (ICH Q5D or regional guidelines). Characterization of the MCB allows the sponsor to assess the source with regard to the presence of cells from other lines, adventitious agents, endogenous agents and molecular contaminants (e.g., toxins or antibiotics from the host organism). A characterization of the criteria for cell banks may include appearance, identity, cell count and viability for cell banks, as well as the sterility, mycoplasma, purity, absence of adventitious viruses and absence of specific human viruses. The objective of this testing is to confirm the identity, purity

and suitability of the cell substrates for manufacturing oncolytic viruses.

Another dimension of cell characterization is the appropriateness for their intended use in oncolytic virus production. There are two concerns for cell substrate stability: the consistent production of the oncolytic virus and the retention of production capacity during storage under defined conditions.

A two-tiered virus banking system, a Master Virus Bank (MVB) and a Working Virus Bank (WVB), is generally constructed for the production of oncolytic virus products. The MVB and WVB should also be characterized and should be subject to extensive quality control; the established guidelines may be applicable to evaluate these banks (ICH Q5D or regional guidelines). A characterization of the criteria for virus banks may include particle number and infectious titer, sterility, mycoplasma, purity, absence of adventitious viruses, replication-competent viruses and molecular variants, and absence of specific human viruses. A MVB is produced from an initial seed virus, and a WVB is derived from one or more containers of the MVB. The MVB and WVB should be produced under optimized culture conditions for viral growth and harvest, and be thoroughly defined, giving an efficient and reproducible downstream purification process. The quality, safety and efficacy of the final formulation of the oncolytic virus in which the virus will be stable for long periods in storage is guaranteed by the establishment of a well-defined virus banking system.

Sponsors are also encouraged to employ state-of-the-art methods and technological improvements in oncolytic virus characterization and testing as they become available, as long as the specificity, sensitivity and precision of the newer methods are at least equivalent to those of existing methods. Since oncolytic virus therapy has been developed only very recently, technologies for the characterization of oncolytic viruses remain to be fully elucidated. There remain a number of technical challenges concerning oncolytic virus testing and product characterization.

Manufacturing of Oncolytic Viruses

The manufacturing method for oncolytic viruses (vectors) must be fully described, including a description of the cells used for the production of the oncolytic viruses, and all relevant data on the name, manufacturing method, pathogenicity, propagation, growth factor dependence, phenotype, tumorigenecity, stability, etc. Changes in the character of the original cells must be clarified and the cultivation method of the cells described, including the medium, serum, antibiotics or other growth factors used. When a packaging cell is used, the manufacturing procedure, selection, identification method and isolation purification method to produce a seed cell strain must be established and characterized and the genetic stability of any sequence inserted into a packaging cell should also be described. The purification method of oncolytic viruses should be described in detail. When scaling up for manufacturing, suitable validation data to describe the contents should be made available. Additionally, descriptions must be included of the preparation and storage method of the MCB and WCB, as well as of the controlling and renewal methods. Finally, tests should be performed to confirm that the cell phenotype between the lots has not changed during the cultivation period. The test period, method and results of any safety tests necessary for quality control should be justified.

Genetic Stability, Replication-Competent Viruses (RCVs) and Molecular Variants

The biological and manufacturing consistency of oncolytic viruses depends primarily on the genetic stability of virus genomes as well as on the nature of the producer cells. A well-defined cell banking system partially ensures the genetic stability of oncolytic viruses during the manufacturing process. Relevant concerns include the generation of replication-competent viruses (RCVs) and molecular variants during manufacturing. RCVs in products can be evaluated by bioamplification assay [35]. Semiquantitative bioamplification systems are used to detect recombination that may occur during manufacturing. These assays are able both to detect contaminating wild-type viruses and to evaluate the genomic stability of an engineered virus; the oncolytic virus product tested in such assays requires multiple passages. Wild-type viruses that contaminate a preparation of engineered oncolytic virus are also typically detected using quantitative polymerase chain reaction (PCR) [36]. When the molecular variants are predicted by recombination, a preparation of engineered oncolytic virus should be tested for molecular variants using quantitative PCR [35].

The selection of the cell substrate is another strategy to minimize the appearance of recombinant RCVs. In the case of adenovirus production, the amount of replication-competent adenovirus (RCA) detected is higher in batches produced in conventional cell lines (e.g., 293 cells [37]) compared to that found in batches produced in recently engineered cell lines (e.g., PER.C6 cells [38]) because of the sequence homology between the engineered adenoviruses and the integrated sequences in the 293 cells. PER.C6 cells are reported to have produced no RCAs in large-scale adenovirus product [39]. A novel cell line, C139 derived from A549 human lung cancer cells, it has been reported that the E1a and E1b coding regions were reduced to their minimal sequences and that native promoters were deleted [40]. Additionally, it has been reported that neither RCAs nor cytopathic effect (CPE)-inducing replication-deficient recombinants are generated during the production of adenoviral vector using C139.

Adventitious Agent Testing

For more information on adventitious agent testing, ICH guidance Q5A: "Guidance on Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin" [41] should be referred if applicable. *In vitro* viral testing should be performed on the MCB, WCB, MVB, WVB, CAL (cells at the limit of *in vitro* cell age used for production) and oncolytic virus products. In such testing, the test sample (for example, MCB or MVB) is inoculated onto various susceptible indicator cell lines such as the human or primate cell line. The choice of cells used would depend on the species of origin of the oncolytic virus and the cell substrate used. In addition, the test would include a measure of both cytopathic and hemadsorbing viruses.

In vivo viral assays should be carried out by inoculating the test sample (MCB, MVB, etc.) into animals such as adult and

suckling mice, and embryonated hen's eggs. Additional testing of guinea pigs, rabbits or monkeys should also be considered. An assay for species-specific viruses should be performed and rodent cell lines used during production should be tested for rodent-specific viruses. If human cell lines are used in the therapeutic product, testing for human pathogens, including cytomegalovirus (CMV), human immunodeficiency virus (HIV) -1 and 2, human T-cell lymphotropic virus (HTLV) 1 and 2, Epstein-Barr virus (EBV), hepatitis B virus (HBV), hepatitis C virus (HCV), B19, and other human viral agents should be performed if appropriate. Human viral agents may be tested using a PCR-based test system. Retroviral contamination in MCB and MVB must be analyzed using reverse transcriptase (RT) assays and electron microscopic analysis.

Adventitious agent testing may be particularly challenging for oncolytic virus products. One strategy is based on the neutralization of the oncolytic virus with a specific antibody prior to testing for adventitious agents both for *in vitro* and *in vivo* assays. This is done to prevent the product from generating false positive results.

Batch Release

Typical release criteria for oncolytic viruses may be based on appearance, identity, virus titer, virus particles, potency, purity (including product-related and process-related impurity), safety (sterility, mycoplasma, endotoxins, adventitious viruses), and characterization.

In general, a standard potency assessment for oncolytic virus products is carried out based on the ratio of virus particle numbers to infectious titers in the final products. For replication-defective adenoviral vectors, the ratio of adenovirus vector particles to infectious titers must be less than 30:1 to satisfy FDA guidelines [31]. At present, however, no specific guidelines exist concerning the acceptable ratio of physical and infectious titers for oncolytic viruses. In addition to measuring tumor cell line killing in an *in vitro* assay, biological characteristics such as viral infectious titer and particles to infectious unit ratio are very useful to ensure batch-to-batch consistency.

Furthermore, it will be necessary to develop standardized testing procedures that will allow the evaluation and comparison of the selectivity, potency and toxicity of oncolytic viruses. If applicable, a wild-type strain may be useful as a positive control in order to allow normalization of infectivity and viral replication capability between different cell types. In the case of adenovirus products, the Adenovirus Type 5 Reference Material established by FDA and Adenovirus Reference Material Working Group can be used to standardize and normalize quantification methods, particle numbers and infectious titers [42].

The following numerical estimation of expressing the selectivity of an oncolytic virus effect in tumor cells compared to a normal cell line has been proposed [43]:

$$\text{Selectivity} = \frac{[\text{effect of oncolytic virus in tumor cell/effect of wt in tumor cell}]}{[\text{effect of oncolytic virus in normal cell/ effect of wt in normal cell}]}$$

"Effect" can be measured in terms of viral yield (or burst size, CPE (IC50 values), viral late protein expression or viral DNA replication level. Progeny production is considered the most relevant for the desired analytical effect [9].

NON-CLINICAL STUDIES

Non-clinical studies of oncolytic virus products are crucial to establish the safety and proof of concept in advance of clinical trials. Since oncolytic viruses have very unique safety issues, such as the emergence of genetic variants and the risk of germline transmission, international harmonized guidelines such as ICH S6 or other documents do not seem to be applicable. The design of non-clinical studies for oncolytic virus products will depend on the type and nature of the specific oncolytic virus product.

In each case, the objective and design of the animal studies, including the type of animal and the reason for selecting it, must be explained. Non-clinical studies should be designed to obtain data that demonstrate the proof-of-principle of oncolytic virus products and that provide biosafety features: target organs for toxicity, risk of shedding, etc. In general, animal models are valuable for testing non-clinical safety and assessing proof of concept, however, they have certain limitations. Some viruses have species-specific susceptibility to viral infection and replication, there may be differential tropism in tumor-xenograft models, and it is impossible to model all aspects of the immune response. Differences in the tissue architecture between animal models and humans are also an important factor, especially with respect to the role of the connective tissue and intermixed normal cells. Nevertheless, animal models are useful to address specific questions such as the choice of a route of administration, biodistribution, safety/toxicity, dose selection and dose regimen. When possible, the selectivity of virus replication has also been studied using *in vivo* models.

Non-clinical safety studies should initially include single-dose toxicity studies, repeated-dose toxicity studies if appropriate, and biodistribution studies, which can incorporate pharmacodynamic-like endpoints. The type and duration of repeated-dose non-clinical safety studies should be considered dependent on the type of oncolytic virus and potential concerns about insertional mutagenesis, for instance.

A quantitative nucleic acid amplification test (NAT) may be used to investigate tissue distribution and the persistence of the oncolytic virus sequence in biodistribution studies. If the administered oncolytic virus sequence is detected in unintended tissues or organs by a NAT assay, this may assist in determining mRNA for the gene product by RT-PCR. Additionally, RT-NAT immunological-based assays may be used to in verify the duration and level of expression of the gene product to detect functional protein.

According to the potential risk of inadvertent germline integration of oncolytic viruses based on the vector type, route of administration and patient population, it may also be necessary to determine whether or not the nucleic acid of the oncolytic virus is incorporated into gonads. The key element in the assessment of inadvertent germline integration is a well-conducted biodistribution study in animal models.

CLINICAL STUDIES AND SAFETY EVALUATION

Due to the complexity of oncolytic virus products and the limited usefulness of animal models, many concerns, including safety issues, remain to be addressed in early-phase clinical studies. These studies must focus on safety and definitions of dose and clinical strategy.

Clinical Pharmacokinetics

With respect to the pharmacokinetics of oncolytic viruses, both quantitative PCR and infectivity assays may be used to monitor patients. In some cases, quantitative monitoring of administered oncolytic virus genomes may provide data supporting viral replication in permissive tissues.

DOSE SELECTION AND DOSE REGIMEN

The dose selection and dose regimen of oncolytic viruses in clinical use should be carefully assessed to ensure their safety and evaluate their toxicity for humans. In one case, a patient who was enrolled in a phase-one clinical trial using replication-deficient adenovirus vector died due to the injection of high-dose vector [44]. The use of replication-competent viruses poses special concerns since the replication of the virus in the patient may lead to an enhanced level of and prolonged exposure to the virus, and thus might increase the risk of virus-induced toxicity. Replication competence of the oncolytic virus does not eliminate the need to perform dose ranging studies to determine an effective dose level.

Viral Shedding and Risks of Contact Person

Since data on viral shedding are limited with respect to oncolytic viruses [29, 45-49], precautions to reduce the risk of exposure of healthcare providers, family members and other patient contacts should be taken. The possibility of virus shedding and the site of shedding may depend on the site and route of administration, dose and replication efficacy of the virus in question. During clinical trials, risk must be monitored not only in the patient but also in the general population. The monitoring of viral shedding and mobilization/recombination with wild-type strains is recommended, and the need to establish long-term follow-up programs must be evaluated. However, all of these measures should take into account the special aspects of oncolytic viruses, such as the disease spectrum and pathogenicity of wild-type strains versus modified oncolytic viruses, the level of pre-existing immunity in the general population, and the ability of the virus to evade the immune system. The onset of tropism-modified versions of some oncolytic viruses requires additional control since the tropism can be narrowed or expanded, and previous clinical experience with non-modified strains is not necessarily relevant.

Schedule for Patient Follow-Up

It is important to establish observation and follow-up schedules for patients, including investigation of the *in vivo* distribution of the administered oncolytic virus, survival and functional expression terms of the gene of interest, symptoms caused by replication-competent viruses or molecular variants, etc. If the oncolytic virus is found to be transiently distributed to the gonads in animal studies, assaying patient semen for the presence of vector may be considered. However, if the patient population is sterile, or if the patient has a severe disease condition with short life expectancy, monitoring of semen samples may not be necessary.

ABBREVIATIONS

CMV = Cytomegalovirus

CPE = Cytopathic effect

E2F = S-phase of cell cycle promoter

EBV = Epstein-Barr virus

EMEA = European Medicines Agency

EOP cells = End of production cells

FDA = Food and Drug Administration

HBV = Hepatitis B virus

HCV = Hepatitis C virus

HIF-1 = Hypoxia-inducible factor-1

HIV = Human immunodeficiency virus

HSV = Herpes simplex virus

HTLV = Human T-cell lymphotropic virus

ICH = International conference on harmonization of technical requirements for registration of pharmaceuticals for human use

MCB = Master cell bank

MVB = Master virus bank

NAT = Nucleic acid amplification test

NDV = Newcastle disease virus

PCR = Polymerase chain reaction

PSA = Prostate-specific antigen

RCA = Replication-competent adenovirus

RCV = Replication-competent virus

RT = Reverse transcriptase

TERT = Telomerase reverse transcriptase

VSV = Vesicular stomatitis virus

WCB = Working cell bank

WVB = Working virus bank

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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

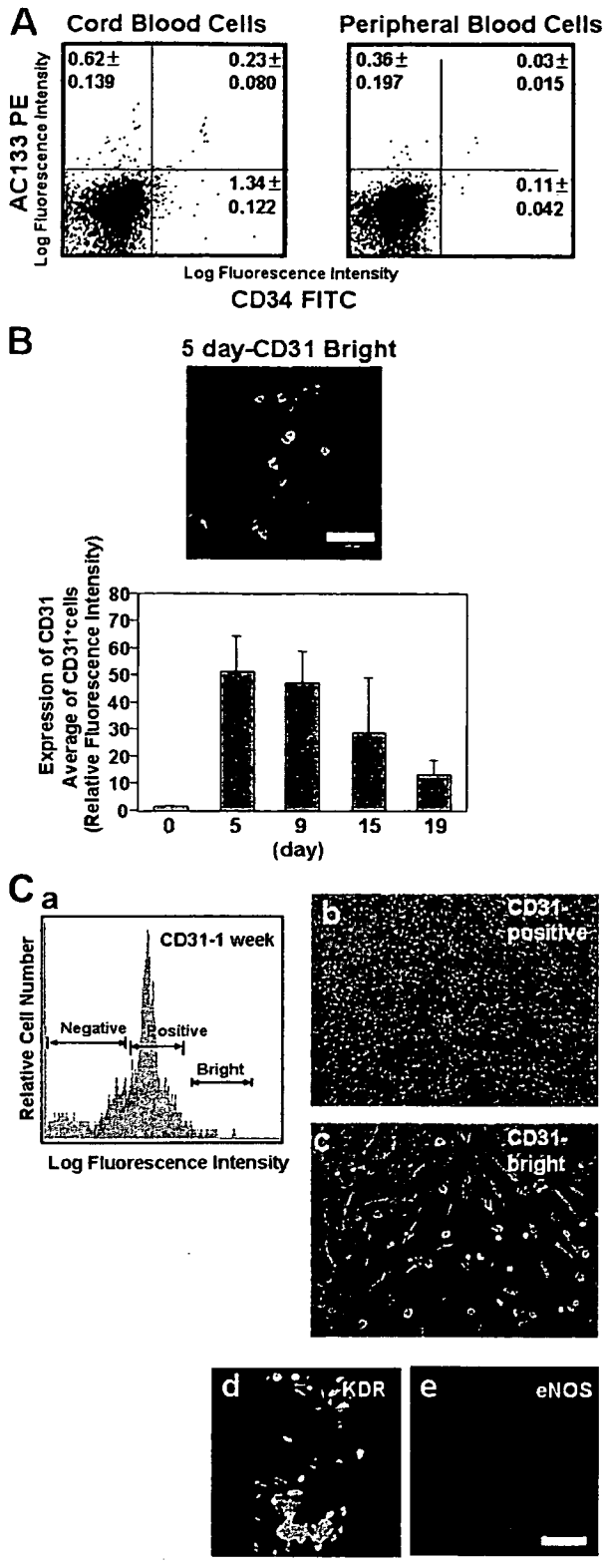

FIGURE 1. ***In vitro* differentiation of AC133$^+$ cells of cord blood into endothelial cells.** *A*, expression of AC133 and CD34 cells in human cord blood and peripheral blood mononuclear cells was analyzed by staining with AC133-PE (*vertical axis*) and CD34-FITC (*horizontal axis*). The *numbers*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

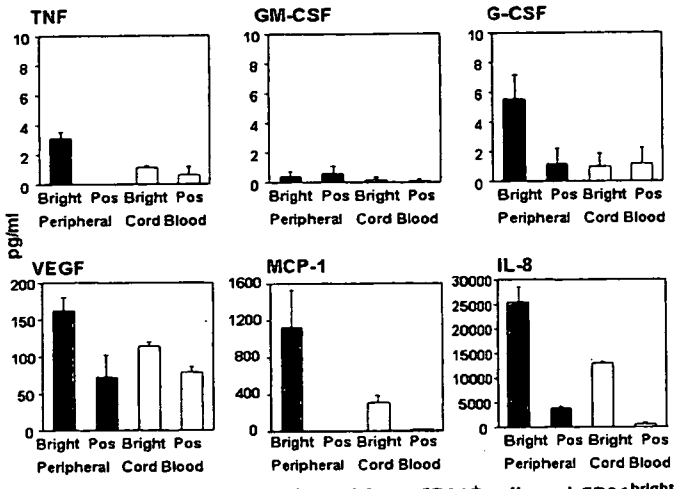

FIGURE 2. **Various cytokines released from CD31$^+$ cells and CD31^{bright} cells.** Production of various cytokines from CD31$^+$ cells and CD31^{bright} cells derived from AC133$^+$ cells cultivated for 5 days was measured. *Gray columns* indicate the cytokine production by cells from peripheral blood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

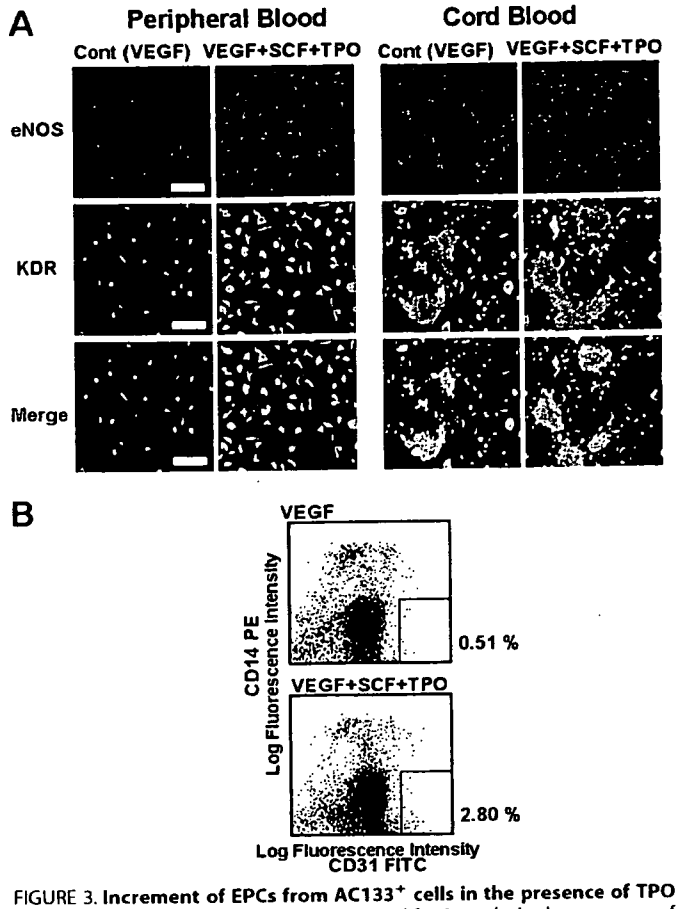

FIGURE 3. **Increment of EPCs from AC133$^+$ cells in the presence of TPO and SCF.** *A*, AC133$^+$ cells were differentiated for 2 weeks in the presence of either VEGF alone or the combination of TPO, SCF, and VEGF on an FN-coated dish. The *upper* and *middle panels* indicate the fluorescent photomicrographs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