科学的・効果的・効率的で柔軟な規制制度を有しているわが国と、第3部をベースにした承認事項の合理化や柔軟な取り扱い、人的資源スリム化を図ろうとしている欧米とは事情が異なる。FDA等の目指す理想の究極に日本型モデル（図2）があるのではないかとの問いに、うなずくFDAの専門家も多い。ところでQ8の一部、Q9、Q10は現在のところオプションである。それらには多くの学ぶべきこともあり、必要に応じて適宜活用するのは望ましいことといえる。一方で、化成品とバイオ医薬品原薬製造に関する共通ガイドラインをQ8、Q9、Q10をバックボーンにしながら作成しようとする動きがある。そのような形で技術的要件のガイドラインができてしまうと、それを通してオプションであるはずのQ8の一部、Q9、Q10が要求事項になってしまうことに思い至る必要がある。ICHは各国に適用できる形で必要な科学的要件の調和を図るべきであろう。

3―遺伝子治療薬や核酸医薬品はこれから

遺伝子治療はまだ安全性・有効性が確立されていないため、重篤な遺伝子疾患、がん、その他の生命を脅かす疾患又は身体の機能を著しく損なう疾患を対象としている。臨床開発初期段階のものが多く、日米欧では未承認であるが、中国等では承認例も出ている。わが国における遺伝子治療研究はいまだ20余例で米国の約840例や欧の約320例に比較して少ない。しかしわが国独自の導入遺伝子としてHGF、ベクターとしてセンダイウイルスベクターやその構成要素を用いた膜融合リポソームの開発などが試みられており、今後の発展に期待したい。遺伝子治療は現在のところ、X連鎖重症複合免疫不全症（X-SCID）、アデノシンデアミナーゼ欠損症、慢性肉芽腫症、パーキンソン病などの単一遺伝子疾患で、著効あるいは一定の効果が得られているが、癌に対する効果は限定的である。一方、X-SCID遺伝子治療でT細胞白血病様症状発症（治療可能）やアデノウイルスベクターの不適切な大量投与による異常免疫反応等の重篤な副作用も見られ、安全性などに慎重な検討が必要である。遺伝子治療用医薬品においては、①複製（増殖）性ウイルスの検出方法、存在許容量と管理方法、②ウイルスタンパク質による抗原性に対する留意と軽減方策、③目的外の細胞・組織への遺伝子導入の回避と投与量の軽減のための方策、④染色体への遺伝子組み込みに伴う遺伝毒性、がん原性発現への慎重な対処と回避策、などが安全性確保上の重要課題であるといわれており、ICHのワーキンググループでも検討対象となっている。

最近ウイルス療法が、がんの治療で遺伝子治療よりも高い効果が期待されるとして注目を浴びてきた。これは、正常細胞内では増殖できず、標的とするがん細胞内でのみ選択的に増殖可能な腫瘍溶解性ウイルス（変異又は組換え単純ヘルペスウイルスや組換えアデノウイルスなど）を用いたがんの新しい治療法である。わが国では4例ほどの実施例がある。

核酸医薬品は、一般にゲノム医学を背景に、特定の遺

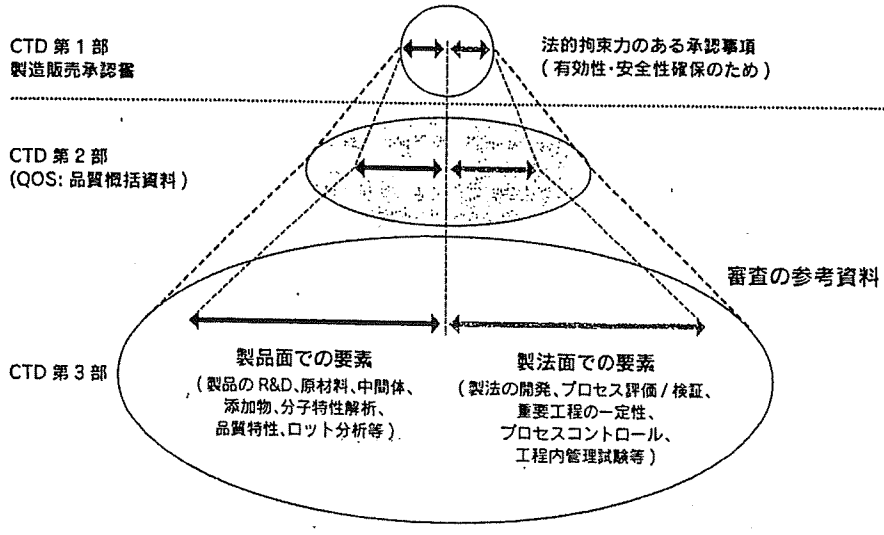

図2　科学に基づく効果的・効率的で柔軟なわが国の品質に関する規制

伝子発現を制御するよう設計した塩基配列を有するものである。アンチセンス、リボザイム、デコイ、siRNA（small interfering RNA）などがこの範疇に含まれる。海外ではサイトメガロウイルス性網膜炎を対象にしたアンチセンスが医薬品となっている。さらに、海外では少なくとも18品目以上のアンチセンス医薬品について臨床試験が行われている。SiRNA医薬品は、海外では少なくとも7品目以上の製品について臨床試験が行われている。リボザイムについてはVEGF受容体、EGF受容体、HCVをターゲットにしたものなどが臨床試験の段階にある。DNAワクチンについては海外で、エイズ、B型肝炎、インフルエンザなどの感染症や、がんを対象とした臨床試験が進められている。

4―日、米、EU、韓国でしのぎをけずる細胞治療

ヒトまたは動物より分離した細胞や組織を培養、加工し、直接患者に投与することによりさまざまな疾患の治療を行うことを一般に細胞治療あるいは再生医療と称している。細胞治療や再生医療に用いられる細胞と対象疾患の関係は、例えば、ガンには活性化リンパ球や樹状細胞等、神経疾患には神経幹細胞、熱傷・創傷等には表皮細胞/線維芽細胞、心筋梗塞には骨格筋芽細胞/心筋芽細胞/血管内皮細胞、リウマチには軟骨芽細胞、骨粗しょう症には骨芽細胞、再生不良性貧血には血液幹細胞、糖尿病にはランゲルハンス細胞、重篤な肝疾患には肝細胞、筋ジストロフィーには筋芽細胞等がある。当面は他の治療法では治療困難な疾患が対象である。わが国では、最近、培養皮膚が製造販売承認を得た。また、外傷性軟骨欠損症などを適応とする軟骨細胞が治験を終了し、さらに心機能改善のための骨格筋芽細胞や造血幹細胞移植時の移植片対宿主病を適応とするヒト間葉系幹細胞が治験準備中である。いわゆる確認申請済みあるいは申請中のものは合わせて6品目である。一方、医師主導型で臨床研究が行われていると推定される例は200例を越える。

最近、再生医療の一層の推進を目指して、確認申請やその審査（先端的治療に用いられる細胞・組織製品における品質・安全性をヒトに投与する前に確認するという、いわゆる上乗せ部分の申請・審査）のあり方についての再検討を含めて、関係指針の見直しが行われている。改訂指針案の概略を表2に示す。

表2　ヒト由来細胞・組織加工医薬品等の品質及び安全性確保に関する改訂指針案の概略

1	自己由来のものと同種(他家)由来のものに分け、それぞれの製品における品質及び安全性確保のために必要な基本的要件を明確にすること。
2	基本的要件は承認申請をも念頭においたものであるのに対して、確認申請とは治験を開始するに当たって支障となる品質、安全性上の問題があるか否かを確認するためという趣旨を踏まえて、基本的要件のうち確認申請までにどの程度の試験や評価をするべきかを明確にすること。
3	従来は必要な試験や評価に関する科学的考え方及び申請に際して必要な情報や記載すべき事項が１つの指針に盛り込まれていたが、確認申請の記載要領に関することは別記事項として明確にする。
4	指針の記述は理解しやすいものとするとともに、Q&Aにより、必要な背景説明を行うこと。
5	細胞・組織加工医薬品等の種類や特性、臨床上の適用法は多種多様であり、また、本分野における科学的進歩や経験の蓄積は日進月歩である。本指針を一律に適用したり、本指針の内容が必要事項すべてを包含しているとみなすことが必ずしも適切でない場合もある。従って、個々の医薬品等についての試験の実施や評価に際しては本指針の目的を踏まえ、その時点の学問の進歩を反映した合理的根拠に基づき、ケース・バイ・ケースの原則で柔軟に対応することが必要であること。
6	最終製品の規格及び試験方法の設定、個別患者への適用ごとの原材料の品質管理、製造工程の妥当性の検証と一定性の維持管理の他、中間製品の品質管理を細胞・組織加工医薬品等の品質管理全体方策の要素ととらえ、これらを相補的、合理的に組合わせて全体として品質管理の目的が達成されるとの観点に立つこと。
7	製品の特性及び適用法から評価が必要と考えられる安全性関連事項について、技術的に可能であれば、科学的合理性のある範囲で、適切な動物を用いた試験又は *in vitro* での試験を実施すること。ヒト由来の試験用検体は貴重であり、また、ヒト由来製品を実験動物等で試験して必ずしも意義ある結果が得られるとは限らない。合理性のない試験の実施を求める趣旨ではないという前提で、製品の特性等を考慮して適切な試験を検討すること。
8	技術的に可能かつ科学的に合理性のある範囲で、実験動物、細胞等を用い、適切に設計された試験により、製品の機能発現、作用持続性、医薬品・医療機器として期待される効果を検討すること。

再生医療分野では、世界の再生医療技術の3分の1を日本発にというスローガンでその推進を図ろうとしている。例えば、角膜再生ではわが国が先行している。皮膚再生、心筋再生、神経再生、膵島再生でわが国は米国あるいはEU等と並んでいる。一方、再生骨・軟骨や体性幹細胞による肝細胞再生技術等では、米国、EUや韓国の後塵を拝している。

ところでヒト胚性幹細胞（ES細胞）はあらゆる細胞に分化誘導可能な万能細胞として注目を浴びてきたが、科学技術的な問題はもとより、倫理的な問題が大きなネックとなってきた。最近、皮膚など正常な組織（細胞）から適切な遺伝子群の導入と適切な増殖因子を組合せる培養により、再プログラム化された人工多能性幹細胞（iPS細胞）が得られることが明らかになった。iPS細胞は、さまざまな基礎研究の対象としてきわめて重要な意義を持つ。その一方で、各種の目的細胞・組織に分化・誘導して、基礎・応用研究に必要な細胞を得ることや、さらに細胞治療・再生医療用の素材としてもきわめて大きな期待を集めている。実用化には、より効率的・効果的なiPS細胞作成技術の開発とその確実性、品質・安全性の確保、iPS細胞の大量獲得技術、各種目的細胞への確実かつ安全な分化・誘導技術と目的細胞の大量生産技術、各段階での細胞の確実な特性解析と最終製品での品質・安全性確保のための評価技術の開発、臨床評価等、切り拓くべき課題は多い。しかし、わが国の本分野の英知を結集して取り組むに価する課題であることは明かである。最終ゴールへの道を明確に示し、推進を図る規制環境のさらなる整備も充実していく必要がある。

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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 × *g* for 10 min, the mitochondria and lysosome fractions were removed by re-centrifugation at 4 °C, 20,000 × *g* for 10 min. The membrane fractions were precipitated by ultracentrifugation at 4 °C, 100,000 × *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-

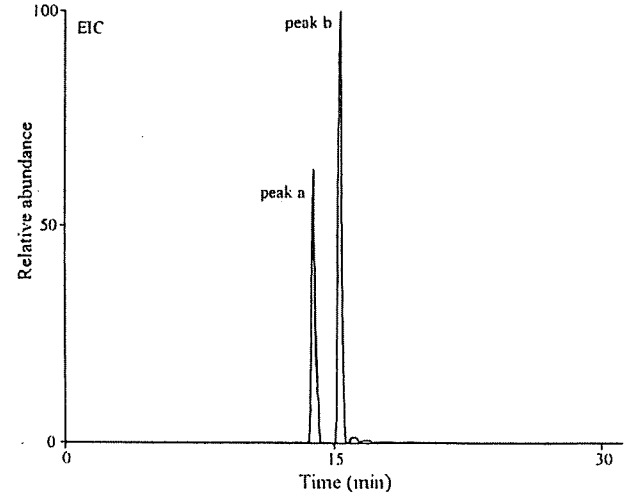

Fig. 1. EIC at *m/z* 426.13–426.17 and *m/z* 442.12–442.16 obtained by SIM (*m/z* 400–450) of DMB-NeuGc and DMB-NeuAc in the positive ion mode.

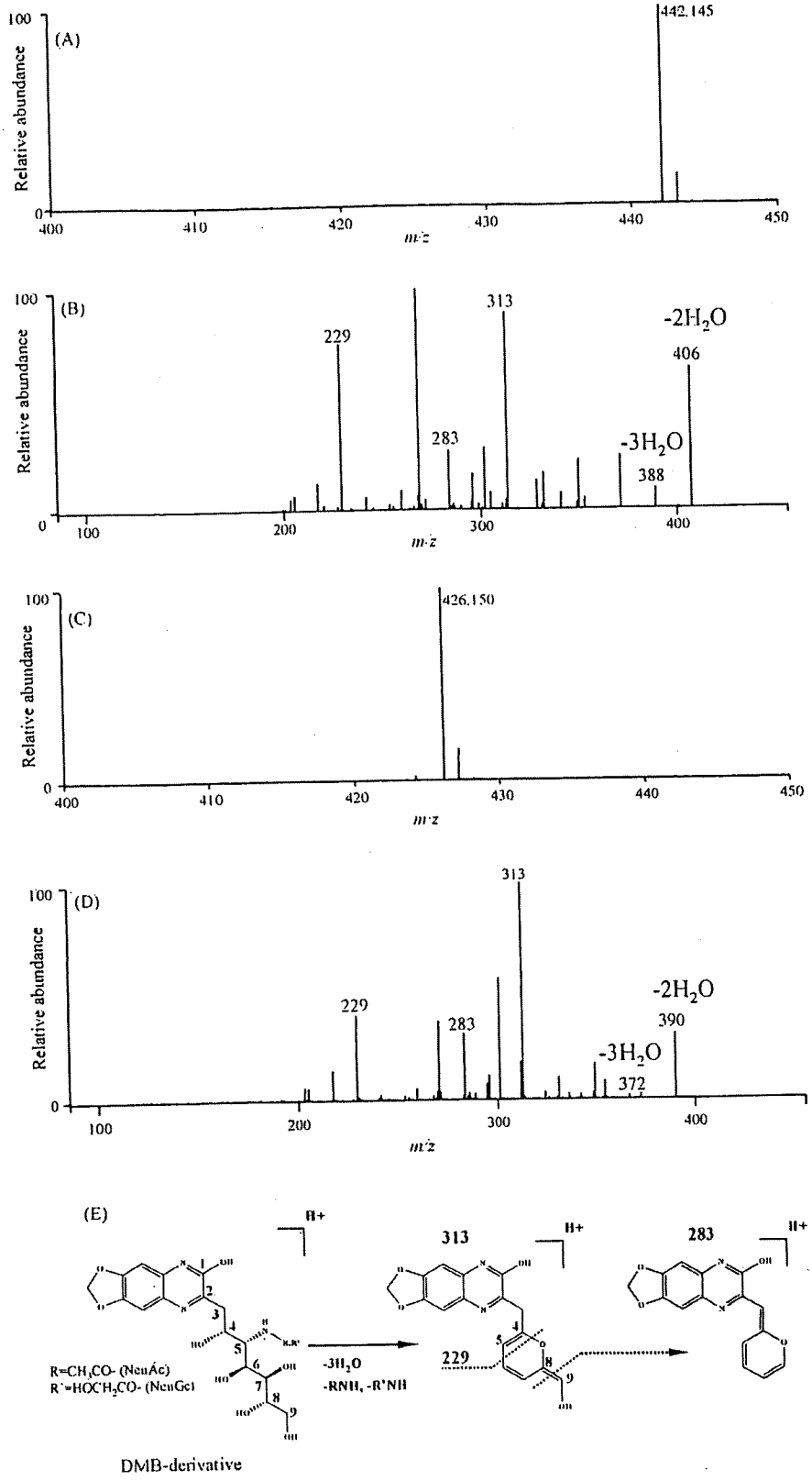

Fig. 2. (A) Typical MS spectrum of peak a. (B) MS/MS spectrum of $[M+H]^+$ (*m/z* 442.145) acquired from around peak a. (C) Typical MS spectrum of peak b. (D) MS/MS spectrum of $[M+H]^+$ (*m/z* 426.150) acquired from around peak b. (E) Fragmentation of DMB-NeuGc and DMB-NeuAc.

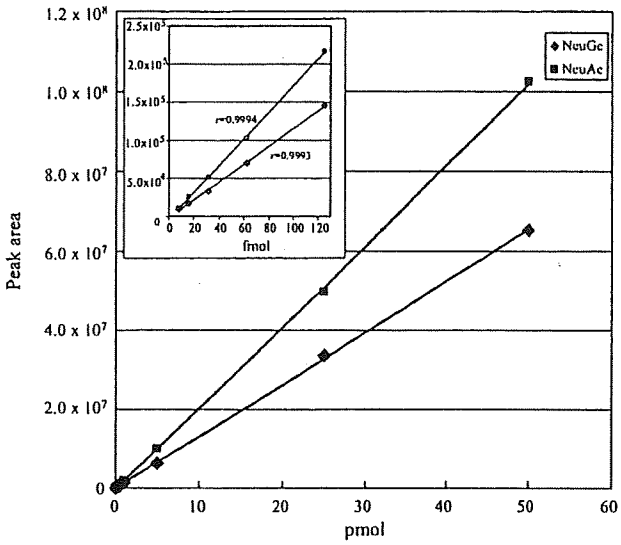

Fig. 3. Calibration curves of DMB-NeuGc ($r = 0.9998$) and DMB-NeuAc ($r = 0.9995$).

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 $DL = 3.3 \times \sigma/\text{slope}$ (σ: average of noise on chromatograph) and $QL = 10 \times \sigma/\text{slope}$, 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.

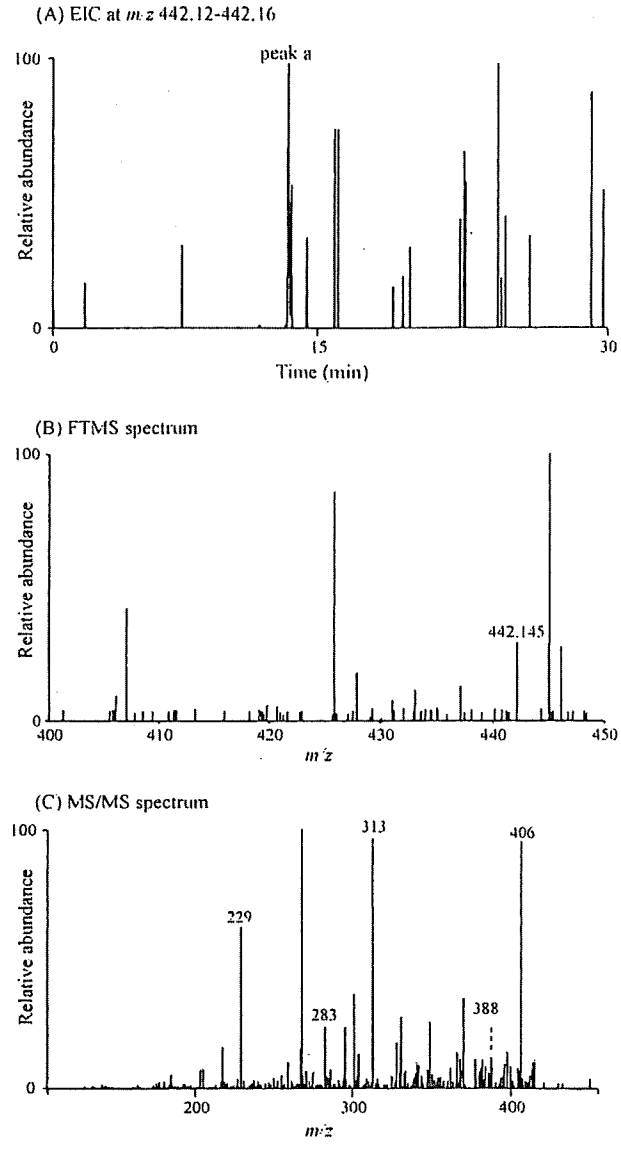

Fig. 4. Detection of DMB-NeuGc in the membrane fractions of HL-60RG cells (2.5×10^3) cultured with 10% FCS. (A) EIC at *m/z* 442.12–442.16 obtained by SIM. (B) Typical MS spectrum of peak a. (C) MS/MS spectrum of $[M+H]^+$ (*m/z* 442.145) acquired from around peak a.

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-

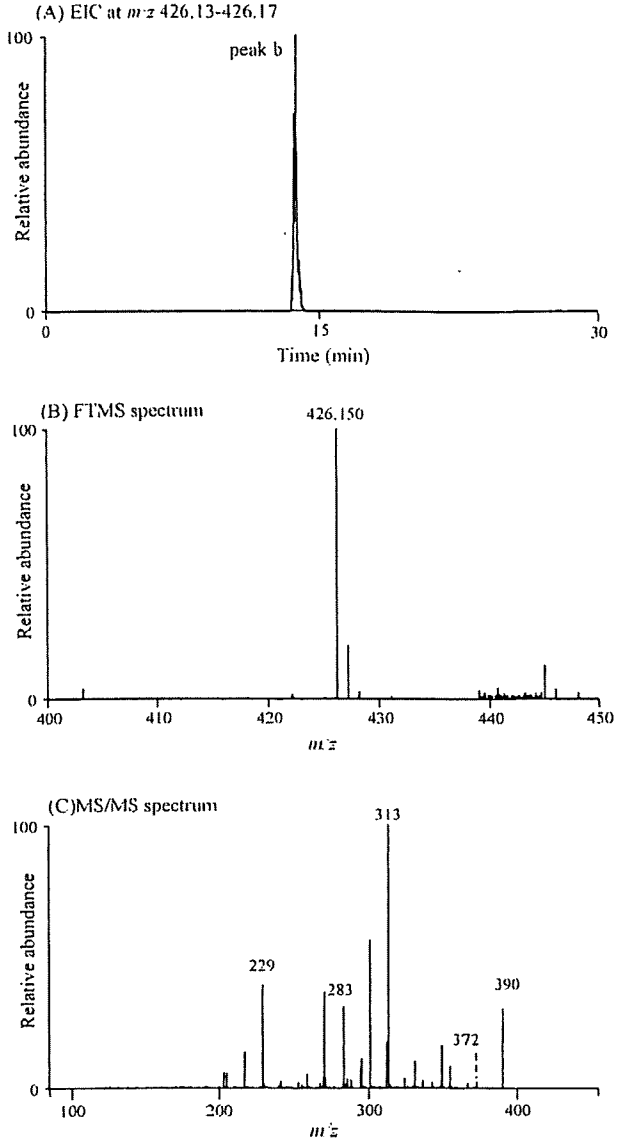

Fig. 5. Detection of DMB-NeuAc in the membrane fractions of HL-60RG cells (2.5×10^3) cultured with 10% FCS. (A) EIC at *m/z* 426.13–426.17 obtained by SIM. (B) Typical MS spectrum of peak b, (C) MS/MS spectrum of $[M+H]^+$ (*m/z* 426.150) acquired from around peak b.

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

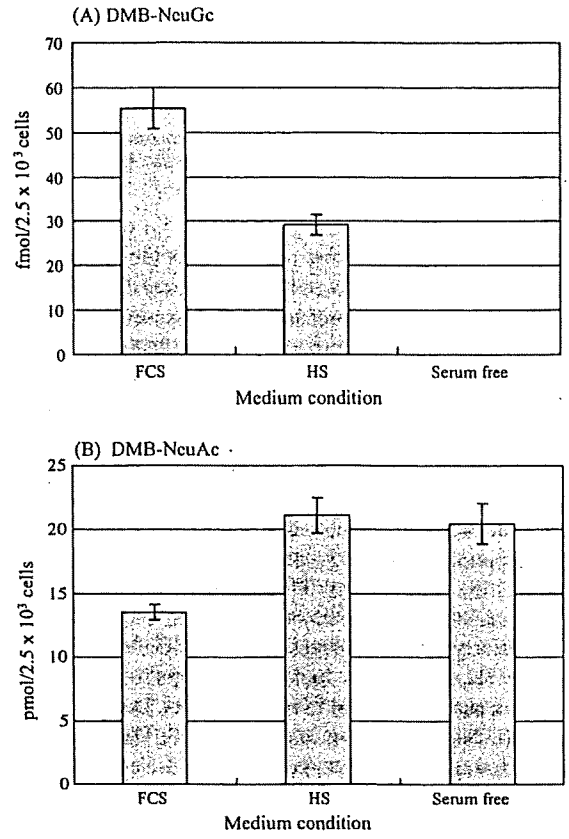

Fig. 6. Levels of (A) NeuGc and (B) NeuAc in the membrane fraction of HL-60RG cells (2.5×10^3) cultured with 10% FCS, 10% human serum (HS) and serum-free medium. Values are the means ± SD ($n = 3$).

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.

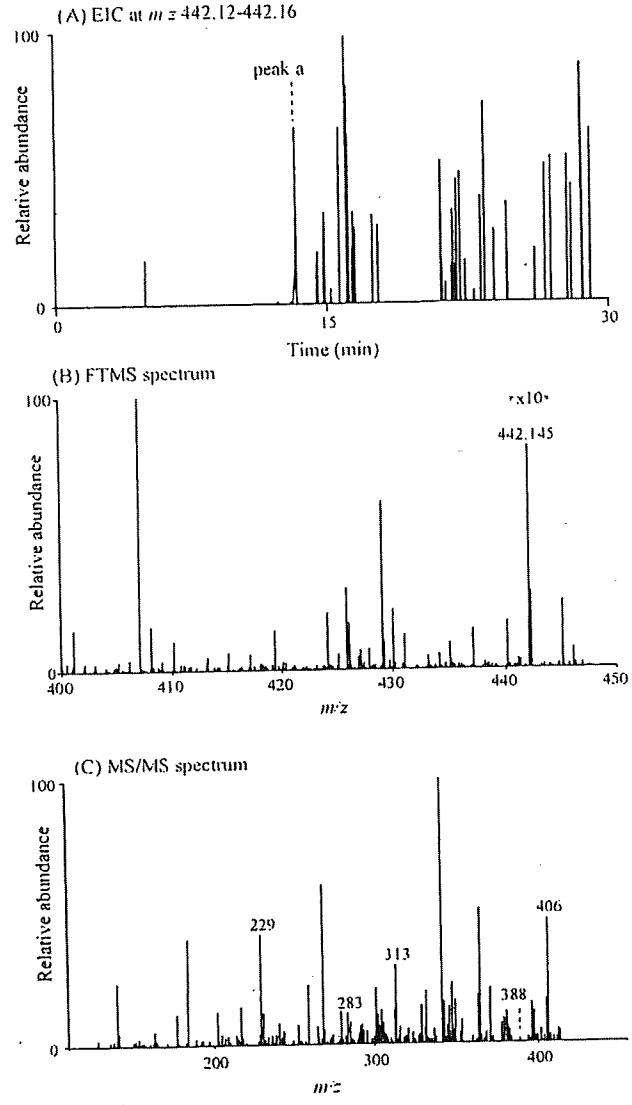

Fig. 7. Detection of DMB-NeuGc in the membrane fractions of HL-60RG cells (2.5×10^3) cultured with 10% human serum. (A) EIC at *m/z* 442.12–442.16 obtained by SIM. (B) Typical MS spectrum of peak a. (C) MS/MS spectrum of $[M+H]^+$ (*m/z* 442.145) acquired from around peak a.

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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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Gene Therapy Discussion Group の動向について**

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Gene Therapy Discussion Groupの動向について**

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1. 横浜会議とこれまでの経緯（Table 1）

遺伝子治療薬は，まだ製品が世の中に出ておらず，ICHにおいても他のEWGと異なる取組みをしています．

本稿では，これまで遺伝子治療専門家会議で行われてきた活動について概説すると共に，横浜会議で取り上げた三つのテーマについて説明します．

一点目は，各極の遺伝子治療に関する最近の進展について，横浜会議で報告し，議論したことがあげられます．

二点目は，この遺伝子治療専門家会議で議論を続けています見解案（Considerations）についてです．横浜会議ではこの「遺伝子治療用医薬品の生殖細胞系列の意図しない伝達リスクを最小にするための方策」見解案のDraft 3の議論を行いました．

三点目は，遺伝子治療の専門家会議で今後取り上げるべき課題についてです．横浜会議で今後の課題について議論し，ステアリングコミッティにいくつかの課題を提案し，了承を得ました．

2. 遺伝子治療について

遺伝子治療とは，様々な遺伝子的疾患あるいは重篤な疾患に対し，目的遺伝子を患者の体内にあるいは体外で患者の細胞に導入して行う治療です（Fig. 1）．

遺伝子治療専門家会議では，例えば新聞で報道されたフランスでの白血病様症状の発症など，有害事象が起きた場合，どのように対処するかも含め，その時点でのサイエンティフィックな到達点に基づき，サイエンスベースのrecommendationを出すといった取組みを行っています．

2.1 遺伝子治療の対象疾患

遺伝子治療の対象疾患は，Table 2に示すように重篤な遺伝性疾患，がん，血管の疾患あるいは神経病などがあげられます．

2.2 遺伝子治療の光と影（Table 3）

遺伝子治療は，当初なかなか治療効果は得られませんでしたが，最近になっていくつかの画期的な成果が得られるようになってきました．

例えば，フランスで行われた重度の免疫不全症であるX連鎖重症複合免疫不全症（X-SCID）に対する造血幹細胞の遺伝子治療において，10人中9人に著効が得られました．この結果，今まで無菌室でしか生活できなかった子供が室外に出られるようになった画期的な治療成果です．その他同じような重度免疫不全症であるアデノシンデアミナーゼ（ADA）欠損症でも有効な治療成績が得られています．更に，最近では白血球による殺菌機能が欠損している慢性肉芽腫症（CGD）という遺伝子疾患についても，遺伝子治療で極めて有望な結果が得られています．

上記三例は先天性遺伝子疾患ですが，これらは導入効率の上昇等の進歩により，非常に著効が得られるようになった事例です．

一方，影の部分として重篤な副作用の発現があります．例えば，アメリカのペンシルベニア大学では1999年に遺伝子治療においてアデノウイルスベクターを所定以上に大量に投与したために死亡した例があり，これを受けて，アデノウイルスベクターの投与量あるいはベクター粒子数の上限が設定されるようになりました．

また，2002年から，フランスのネッカー病院で，レトロウィルスベクターを用いたX-SCID遺伝子

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** 当協会主催の第14回ICH即時報告会（平成18年7月26日）における講演による．

Table 1　遺伝子治療専門家会議横浜会議とこれまでの経緯

- 遺伝子治療専門家会議のこれまでの活動
- 横浜会議で取り上げられたテーマ
  - 各極の遺伝子治療に関する進展
  - ICH見解案「遺伝子治療用医薬品の生殖細胞系列への意図しない伝達リスクを最小にするための方策」Draft3の議論
  - 今後取り上げるべき課題

Table 2　遺伝子治療の対象疾患

重篤な遺伝性疾患，がんその他の生命を脅かす疾患又は身体の機能を著しく損なう疾患

- 先天性遺伝子疾患（単一遺伝子疾患）：ADA欠損症，X-SCID，血友病，筋ジストロフィーなど
- ガン：肺ガン，腎ガン，前立腺ガン，食道ガン，脳腫瘍，黒色腫など
- 末梢性血管疾患：閉塞性動脈硬化症など
- 虚血性心疾患：狭心症，心筋梗塞など
- 神経変性疾患：アルツハイマー病，パーキンソン病，筋萎縮性側索硬化症（ALS）など
- ウイルス感染症：HIV，B型，C型肝炎ウイルスなど
- 生活習慣病，慢性疾患：糖尿病，関節リウマチなど

Table 3　遺伝子治療の光と影

成功例

- X連鎖重症複合免疫不全症（X-SCID）に対する造血幹細胞遺伝子治療（レトロウイルスベクターでIL-2Rコモンγ鎖を導入）により10人中9人に著効
- アデノシンデアミナーゼ欠損症（ADA-SCID）に有効
- 慢性肉芽腫症（CGD）の遺伝子治療で極めて有望な結果

重篤な副作用の発現

- 1999年　アデノウイルスベクターの投与による異常免疫反応により死亡（米・ペンシルベニア大）
- 2002年　レトロウイルスベクターによるX-SCID遺伝子治療で遺伝子の染色体挿入が原因となり3名にT細胞白血病様症状発症（仏・ネッカー病院）
- 遺伝子治療はまだ医療として充分に確立しておらず，有効性，安全性を慎重に検討する必要がある

治療で，遺伝子の染色体挿入が原因となり3名にT細胞白血病様症状が発症しました．

このような重篤な有害事象が起こることもあり，遺伝子治療はまだ医療として十分に確立していないといえます．このため，有効性，安全性を慎重に検討する必要があるとの姿勢で遺伝子治療専門家会議は行われています．

3．ICH遺伝子治療専門家会議（GTDG）

2001年5月のICHのステアリングコミッティにおいて，遺伝子治療薬などの製品の規制に重大な影響を及ぼす可能性がある新しい科学的知見に関する情報について，ICH各極間での情報の交換及び共有を積極的に継続して行う必要があるとの認識のも

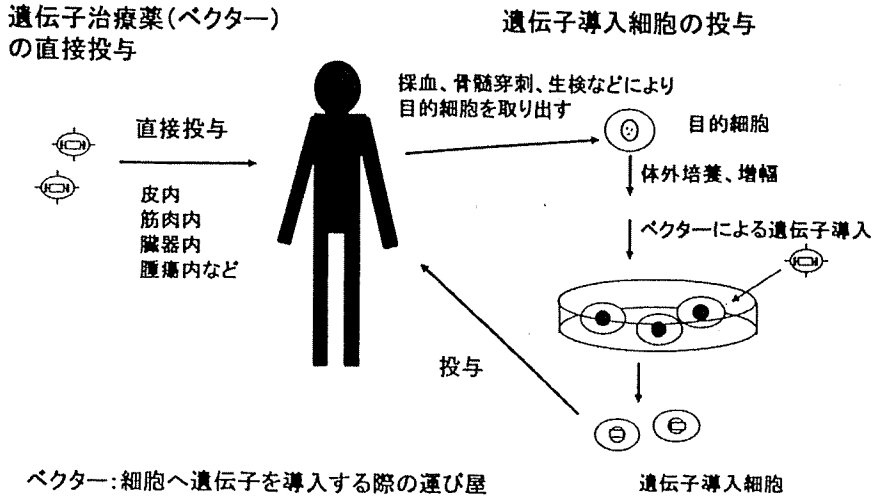

Fig. 1　遺伝子治療とは

と，遺伝子治療専門家会議 (Gene Therapy Discussion Group; GTDG) が ad hoc に新設されました．他の専門家作業グループのように EWG ではない理由は，EWG はガイドラインを作る時に立ち上げる専門家作業グループですが，現時点ではガイドラインに取り上げる取組みをしていないため，遺伝子治療専門家会議となりました．現時点での参加メンバー（Table 4）には，ICH 3極6グループに EFTA とカナダが加わっています．

3.1　遺伝子治療専門家会議の活動（Table 5）

1997年及び2001年の ICH 会議のバイオテクノロジー専門家会議において遺伝子治療の問題が取り上げられ，2002年に初めて遺伝子治療専門家会議が正式に発足しました．その後2003年は大阪での ICH6，2004年はワシントン，2005年はブリュッセルとシカゴで遺伝子治療専門家会議が開催されました．

3.2　遺伝子治療専門家会議の目的（Table 6）

遺伝子治療専門家会議の目的の一つは，遺伝子治療分野は非常に急速に進展しているため，その科学的事項について調査・検討することです．

二つ目は，遺伝子治療用医薬品に関する規制の国際調和に有益な影響をもたらす可能性のある一般的原則をあらかじめ公表することです．

三つ目は，ICH における議論の成果が社会に広く浸透し，十分理解されることを保証するための社会に向けた新しいコミュニケーション手段として，インターネット等を利用して公開することです．

例えば2002年，2003年及び2005年に公開ワークショップを開催しています．2005年は，後述する腫瘍溶解性ウイルスに関する公開ワークショップを開催しています．

また，ICH のステアリングコミッティで遺伝子治療専門家会議の公式声明を発表し，その時点での到達点を公開します．更に誰でもアクセス可能な ICH 遺伝子治療のホームページを開設し，これを ICH 事務局のホームページ内に開設する了解を得て公開しています．国立医薬品食品衛生研究所（衛研）の遺伝子細胞医薬部のホームページでは，これを日本語に仮訳して掲載しています．更にこの衛研のホー

Table 4　ICH遺伝子治療専門家会議参加メンバー

Klaus Cichutek (EMEA), Stephanie Simek (FDA), Teruhide Yamaguchi (MHLW), Christine-Lise Julou (EFPIA), Wataru Toriumi (JPMA), Alex Kuta (PhRMA), EFTA, Canada

Table 5　ICH遺伝子治療専門家会議の活動

1997年ブラッセル： 　バイオテクノロジー専門家会議
2001年 東京・舞浜： 　バイオテクノロジー専門家会議
2002年ワシントン： 　遺伝子治療専門家会議として正式に発足
2003年大阪（ICH6）：遺伝子治療専門家会議
2004年ワシントン：遺伝子治療専門家会議
2005年ブラッセル：遺伝子治療専門家会議
2005年シカゴ：遺伝子治療専門家会議

Table 6　ICH遺伝子治療専門家会議の目的

- 研究が進められている科学的事項について調査・検討
- 遺伝子治療用医薬品に関する規制の国際調和に有益な影響をもたらす可能性のある一般的原則を予め積極的に提示
- ICH における議論の成果が社会に広く浸透し，十分理解されることを保証するための，社会に向けた新しいコミュニケーション手段を開発
  - 例：ICH 遺伝子治療公開ワークショップの開催
    - → 2002年9月，2003年11月，2005年11月に開催
      ICH SC を介しての ICH GTDG 公的声明の発表
      誰でもアクセス可能な ICH 遺伝子治療ホームページの開設
    - → ICH 事務局ホームページ内（英語）：
      www.ich.org/cache/html/1386-272-1.html
      国立衛研 遺伝子細胞医薬部ホームページ内（日本語）：
      www.nihs.go.jp/cgtp/cgtp/sec1/index1-j.html