

Granulocyte Colony-Stimulating Factor Promotes the Translocation of Protein Kinase C ζ in Neutrophilic Differentiation Cells

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Previously, we suggested that the phosphatidylinositol 3-kinase (PI3K)-p70 S6 kinase (p70 S6K) pathway plays an important role in granulocyte colony-stimulating factor (G-CSF)-dependent enhancement of the neutrophilic differentiation and proliferation of HL-60 cells. While atypical protein kinase C (PKC) has been reported to be a regulator of p70 S6K, abundant expression of PKC ζ was observed in myeloid and lymphoid cells. Therefore, we analyzed the participation of PKC ζ in G-CSF-dependent proliferation. The maximum stimulation of PKC ζ was observed from 15 to 30 min after the addition of G-CSF. From 5 to 15 min into this lag time, PKC ζ was found to translocate from the nucleus to the membrane. At 30 min it re-translocated to the cytosol. This dynamic translocation of PKC ζ was also observed in G-CSF-stimulated myeloperoxidase-positive cells differentiated from cord blood cells. Small interfering RNA for PKC ζ inhibited G-CSF-induced proliferation and the promotion of neutrophilic differentiation of HL-60 cells. These data indicate that the G-CSF-induced dynamic translocation and activation processes of PKC ζ are important to neutrophilic proliferation.

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Hematopoietic cell differentiation is regulated by a complex network of growth and differentiation factors (Tenen et al., 1997; Ward et al., 2000). Granulocyte colony-stimulating factor (G-CSF) and its receptors are pivotal to the differentiation of myeloid precursors into mature granulocytes. In previous studies (Kanayasu-Toyoda et al., 2002) on the neutrophilic differentiation of HL-60 cells treated with either dimethyl sulfoxide (DMSO) or retinoic acid (RA), heterogeneous transferrin receptor (Trf-R) populations—transferrin receptor-positive (Trf-R⁺) cells and transferrin receptor-negative (Trf-R⁻) cells—appeared 2 days after the addition of DMSO or RA. The Trf-R⁺ cells were proliferative-type cells that had higher enzyme activity of phosphatidylinositol 3-kinase (PI3K) and protein 70 S6 kinase (p70 S6K), whereas the Trf-R⁻ cells were differentiation-type cells of which Tyr705 in STAT3 was much more phosphorylated by G-CSF. Inhibition of either PI3K by wortmannin or p70 S6K by rapamycin was found to eliminate the difference in differentiation and proliferation abilities between Trf-R⁺ and Trf-R⁻ cells in the presence of G-CSF (Kanayasu-Toyoda et al., 2002). From these results, we concluded that proteins PI3K and p70 S6K play important roles in the growth of HL-60 cells and negatively regulate neutrophilic differentiation. On the other hand, the maximum kinase activity of PI3K was observed at 5 min after the addition of G-CSF (Kanayasu-Toyoda et al., 2002) and that of p70 S6K was observed between 30 and 60 min after, indicating a lag time between PI3K and p70 S6K activation. It is conceivable that any signal molecule(s) must transduce the G-CSF signal during the time lag between PI3K and p70 S6K. Chung et al. (1994) also showed a lag time between PI3K and p70 S6K activation on HepG2 cells stimulated by platelet-derived growth factor (PDGF), suggesting that some signaling molecules also may transduce between PI3K and p70 S6K.

Protein kinase C (PKC) is a family of Ser/Thr kinases involved in the signal transduction pathways that are triggered by numerous extracellular and intracellular stimuli. The PKC

family has been shown to play an essential role in cellular functions, including mitogenic signaling, cytoskeleton rearrangement, glucose metabolism, differentiation, and the regulation of cell survival and apoptosis. Eleven different members of the PKC family have been identified so far. Based on their structural similarities and cofactor requirements, they have been grouped into three subfamilies: (1) the classical or conventional PKCs (cPKC α , β ₁, β ₂, and γ), activated by Ca²⁺, diacylglycerol, and phosphatidyl-serine; (2) the novel PKCs (nPKC δ , ϵ , η , and θ), which are independent of Ca²⁺ but still responsive to diacylglycerol; and (3) the atypical PKCs (aPKC ζ and ι/λ), where PKC λ is the homologue of human PKC ζ . Atypical PKCs differ significantly from all other PKC family

Abbreviations: DMSO, dimethyl sulfoxide; fMLP-R, formyl-Met-Leu-Phe receptor; RA, retinoic acid; G-CSF, granulocyte colony-stimulating factor; Trf-R, transferrin receptor; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; PKC, protein kinase C; PI3K, phosphatidylinositol 3-kinase; p70 S6K, protein 70 S6 kinase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; siRNA, small interfering RNA; PMN, polymorphonuclear leukocyte.

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members in their regulatory domains, in that they lack both the calcium-binding domain and one of the two zinc finger motifs required for diacylglycerol binding (Liu and Heckman, 1998). Romanelli et al. (1999) reported that p70 S6K is regulated by PKC ζ and participates in a PI3K-regulated signaling complex. On the other hand, Selbie et al. (1993) reported that the tissue distribution of PKC ζ is different from that of PKC λ , and that PKC λ appears to be widely expressed. If the p70 S6K could be activated by aPKC, the regulation of p70 S6K activation would seem to depend on the tissue-specific expression of PKC λ and/or PKC ζ . In neutrophilic lineage cells, the question is which aPKC participates in the regulation of p70 S6K on G-CSF signaling. In this study, we show that G-CSF activated PKC λ , promoting its translocation from the nucleus to the cell surface membrane and subsequently to the cytosol in DMSO-treated HL-60 cells. We also show the translocation of PKC λ using myeloperoxidase-positive neutrophilic lineage differentiated from cord blood, which is a rich source of immature myeloid cells (Fritsch et al., 1993; Rappold et al., 1997; Huang et al., 1999; Debili et al., 2001; Hao et al., 2001). We concluded that PKC λ translocation and activation by G-CSF are needed for neutrophilic proliferation.

Materials and Methods

Reagents

Anti-p70 S6K polyclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-PKC λ polyclonal antibody and monoclonal antibody were purchased from Santa Cruz Biotechnology and from Transduction Laboratories (Lexington, KY), respectively. Anti-PKC ζ polyclonal antibody was purchased from Cell Signaling Technology (Beverly, MA). Anti-myeloperoxidase antibody was purchased from Serotec Ltd. (Oxford, UK), GF 109203X, and G66983 were obtained from Calbiochem-Novabiochem (San Diego, CA). Wortmannin was obtained from Sigma Chemical (St. Louis, MO). Anti-Histone-H1 antibody, anti-Fc γ receptor 1a (CD32) antibody, and anti-lactate dehydrogenase antibody were from Upstate Cell Signaling Solutions (Lake Placid, NY), Lab Vision Corp. (Fremont, CA), and Chemicon International, Inc. (Temecula, CA), respectively.

Cell culture

HL-60, Jurkat, K562, U937, and THP-1 cells were kindly supplied by the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). Cells were maintained in RPMI 1640 medium containing 10% heat-inactivated FBS and 30 mg/L kanamycin sulfate at 37°C in moisturized air containing 5% CO $_2$. The HL-60 cells, which were at a density of 2.5×10^5 cells/ml, were differentiated by 1.25% DMSO. Two days after the addition of DMSO, the G-CSF-induced signal transduction was analyzed using either magnetically sorted cells or non-sorted cells.

Magnetic cell sorting

To prepare Trf-R $^-$ and Trf-R $^+$ cells, magnetic cell sorting was performed as previously reported (Kanayasu-Toyoda et al., 2002), using an automatic cell sorter (AUTO MACS; Miltenyi Biotec, Bergisch Gladbach, Germany). After cell sorting, both cell types were used for Western blotting and PKC λ enzyme activity analyses.

Preparation of cell lysates and immunoblotting

For analysis of PKC λ and PKC ζ expression, a PVDF membrane blotted with 50 μ g of various tissues per lane was purchased from BioChain Institute (Hayward, CA). Both a polymorphonuclear leukocytes (PMNs) fraction and a fraction containing lymphocytes and monocytes were isolated by centrifugation (400g, 25 min) using a Mono-poly resolving medium (Dai-Nippon Pharmaceutical, Osaka, Japan) from human whole blood, which was obtained from a healthy volunteer with informed consent. T-lymphocytes were further isolated from the mixture fraction using the Pan T Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's protocol. T-lymphocytes, PMNs, HL-60 cells, Jurkat cells, K562 cells, and U937 cells (1×10^7) were

collected and lysed in lysis buffer containing 1% Triton X-100, 10 mM K $_2$ HPO $_4$ /KH $_2$ PO $_4$ (pH 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 cocktail (Sigma Chemical) and 1/100 (v/v) phosphatase inhibitor cocktail (Sigma Chemical). The cellular lysate of 10^6 cells per lane was subjected to Western blotting analysis. Human cord blood was kindly supplied from the Metro Tokyo Red Cross Cord Blood Bank (Tokyo, Japan) with informed consent. Mononuclear cells, isolated with the Lymphoprep $^{\text{TM}}$ tube (Axis-Shield PoC AS, Oslo, Norway), were cultured in RPMI 1640 medium containing 10% FBS in the presence of G-CSF for 3 days. Cultured cells were collected, and the cell lysate was subjected to Western blotting analysis.

A fraction of the plasma membrane, cytosol, and nucleus of the DMSO-treated HL-60 cells was prepared by differential centrifugation after the addition of G-CSF, as described previously (Yamaguchi et al., 1999). After the cells that had been suspended in 250 mM sucrose/10 mM Tris-HCl (pH 7.4) containing 1/100 (v/v) protease inhibitor cocktail (Sigma Chemical) were gently disrupted by freezing and thawing, they were centrifuged at 800g, 4°C for 10 min. The precipitate was suspended in 10 mM Tris-HCl (pH 6.7) supplemented with 1% SDS. It was then digested by benzonuclease at 4°C for 1 h and used as a sample of the nuclear fraction. After the post-nuclear supernatant was re-centrifuged at 100,000 rpm (452,000g) at a temperature of 4°C for 40 min, the precipitate was used as a crude membrane fraction and the supernatant as a cytosol fraction. Western blotting analysis was then performed as described previously (Kanayasu-Toyoda et al., 2002). The bands that appeared on x-ray films were scanned, and the density of each band was quantitated by Scion Image (Scion, Frederick, MD) using the data from three separate experiments.

Kinase assay

The activity of PKC λ was determined by phosphorous incorporation into the fluorescence-labeled pseudosubstrate (Pierce Biotechnology, Rockford, IL). The cell lysates were prepared as described above and immunoprecipitated with the anti-PKC λ antibody. Kinase activity was measured according to the manufacturer's protocol. In the analysis of inhibitors effects, cells were pretreated with a PI3K inhibitor, wortmannin (100 nM), or PKC inhibitors, GF109207X (10 μ M) and G66983 (10 μ M) for 30 min, and then stimulated by G-CSF for 15 min.

Observation of confocal laser-scanning microscopy

Upon the addition of G-CSF, PKC λ localization in the DMSO-treated HL-60 cells for 2 days was examined by confocal laser-activated microscopy (LSM 510, Carl Zeiss, Oberkochen, Germany). The cells were treated with 50 ng/ml G-CSF for the indicated periods and then fixed with an equal volume of 4.0% paraformaldehyde in PBS(-). After treatment with ethanol, the fixed cells were labeled with anti-PKC λ antibody and with secondary antibody conjugated with horseradish peroxidase. They were then visualized with TSA $^{\text{TM}}$ Fluorescence Systems (PerkinElmer, Boston, MA).

Mononuclear cells prepared from cord blood cells were cultured in RPMI 1640 medium containing 10% FBS in the presence of G-CSF for 7 days. Then, for serum and G-CSF starvation, cells were cultured in RPMI 1640 medium containing 1% BSA for 11 h. After stimulation by 50 ng/ml G-CSF, the cells were fixed, stained with both anti-PKC λ polyclonal antibody and anti-myeloperoxidase monoclonal antibody, and finally visualized with rhodamine-conjugated anti-rabbit IgG and FITC-conjugated anti-mouse IgG, respectively.

RNA interference

Two pairs of siRNAs were chemically synthesized: annealed (Dharmacon RNA Technologies, Lafayette, CO) and transfected into HL-60 cells using Nucleofector $^{\text{TM}}$ (Amaxa, Cologne, Germany). The sequences of sense siRNAs were as follows: PKC λ , GAAGAAGCCUUUAGACUUUUA; p70 S6K, GCAAGGAGUCUUAUCCAUAGUU. As a control, the sequence ACUCUUAUCGCCAGCGUGACUU was used. Forty-eight hours after treatment with siRNA, the cells were lysed for Western blot analysis. For proliferation and differentiation assay, cells were transfected with siRNA on the first day, treated with DMSO on the second day, and supplemented with G-CSF on the third day. After cells were subsequently cultured for 5 days, cell numbers and formyl-Met-Leu-Phe receptor (fMLP-R) expression were determined.

fMLP-R expression

The differentiated cells were collected and incubated with FITC-conjugated fMLP; then, labeled cells were subjected to flow cytometric analysis (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ).

Statistical analysis

Statistical analysis was performed using unpaired Student's *t*-test. Values of *P* < 0.05 were considered to indicate statistical significance. Each experiment was repeated at least three times and representative data were indicated.

Results

The distribution of atypical PKC in various tissues and cells

Previously, we reported that the PI3K-p70 S6K-cMyc pathway plays an important role in the G-CSF-induced proliferation of DMSO-treated HL-60 cells, not only by enhancing the activity of both PI3K and p70 S6K but also by inducing the c-Myc protein (Kanayasu-Toyoda et al., 2002, 2003). We also reported that G-CSF did not stimulate Erk1, Erk2, or 4E-binding protein 1. The maximum kinase activity of PI3K was observed 5 min after the addition of G-CSF, and that of p70 S6K was observed between 30 and 60 min after. It is conceivable that any signal molecule(s) must transduce the G-CSF signal during the time lag between PI3K and p70 S6K. Romanelli et al. (1999) suggested that the activation of p70 S6K is regulated by PKC ζ and participates in the PI3K-regulated signaling complex. To examine the role of atypical PKC in the G-CSF-dependent activation and the relationship between atypical PKC and p70 S6K, the protein expression of PKC ζ and PKC ι in various human tissues and cells was analyzed by Western blotting. As shown in Figure 1A, both of the atypical PKCs were markedly expressed in lung and kidney but were weakly expressed in spleen, stomach, and placenta. In brain, cervix, and uterus, the expression of only PKC ι was observed. Selbie et al. (1993) have reported observing the expression of PKC ζ not in protein levels but in RNA levels, in the kidney, brain, lung, and testis, and that of PKC ι in the kidney, brain, and lung. In this study, the protein expression of PKC ι in the kidney, brain, and lung was consistent with the RNA expression of PKC ι . Despite the strong expression of PKC ζ RNA in brain (Selbie et al., 1993), PKC ζ protein was scarcely observed. Although PKC ι proteins were scarcely expressed in neutrophils and T-lymphocytes in peripheral blood, they were abundantly expressed in immature blood cell lines, that is, Jurkat, K562, U937, and HL-60 cells (Fig. 1B), in contrast with the very low expression of PKC ζ proteins. In mononuclear cells isolated from umbilical cord blood, which contains large numbers of immature myeloid cells and has a high proliferation ability, the expression of PKC ι proteins was also observed. Since Nguyen and Dessauer (2005) have reported observing abundant PKC ζ proteins in THP-1 cells, as a positive control for PKC ζ , we also performed a Western blot of THP-1 cells (Fig. 1B, right part). While PKC ι was markedly expressed in both THP-1 and HL-60 cells, PKC ζ was observed only in THP-1 cells. These data suggested that PKC ζ and PKC ι were distributed differently in various tissues and cells, and that mainly PKC ι proteins were expressed in proliferating blood cells.

Stimulation of PKC ι activity by G-CSF

Among the 11 different members of the PKC family, the α PKCs (ζ and ι) have been reported to activate p70 S6K activity and to be regulated by PI3K (Akimoto et al., 1998; Romanelli et al., 1999). As shown in Figure 1, although the PKC ζ proteins were not detected by Western blotting in HL-60 cells or mononuclear cells isolated from cord blood cells, it is possible that PKC ι could functionally regulate p70 S6K as an upstream

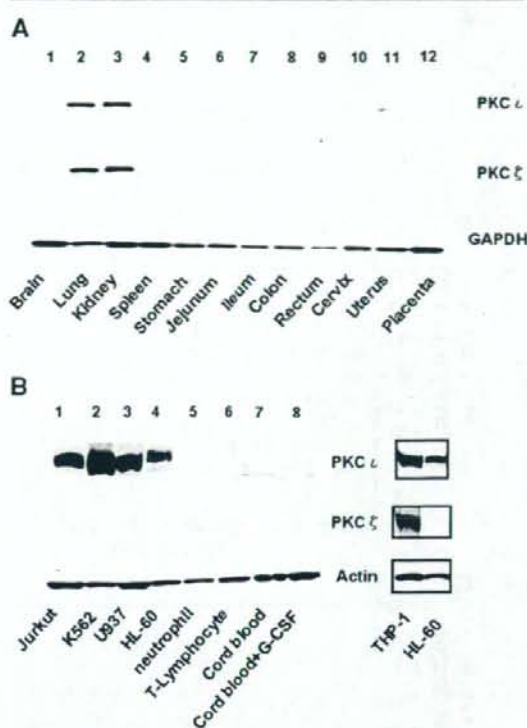


Fig. 1. Different distributions of PKC ζ and PKC ι . The protein expression of PKC ι appears in the upper part and that of PKC ζ in the middle part in various tissues and cells. A: 1, brain; 2, lung; 3, kidney; 4, spleen; 5, stomach; 6, jejunum; 7, ileum; 8, colon; 9, rectum; 10, cervix; 11, uterus; 12, placenta. Anti-GAPDH blot is a control for various tissues. B: 1, Jurkat cells; 2, K562 cells; 3, U937 cells; 4, HL-60 cells; 5, neutrophils; 6, T-lymphocytes; 7, mononuclear cells from cord blood in the absence of G-CSF; 8, mononuclear cells from cord blood in the presence of G-CSF. Anti-actin blot is a control. The right part shows immunoblots of PKC ζ , PKC ι , and actin of THP-1 cells as a positive control for PKC ζ . The cell numbers of THP-1 and HL-60 cells were adjusted in relation to other cells on the left parts.

regulator in these cells. Therefore, we focused on the role of PKC ι as the possible upstream regulator of p70 S6K in neutrophil lineage cells. First, we compared the expression of PKC ι in both Trf-R $^{+}$ and Trf-R $^{-}$ cells. PKC ι proteins were expressed more abundantly in Trf-R $^{+}$ cells than in Trf-R $^{-}$ cells (Fig. 2A, middle part), as with the p70 S6K proteins. A time course study of PKC ι activity upon the addition of G-CSF revealed the maximum stimulation at 15 min, lasting until 30 min. The G-CSF-dependent activation of PKC ι was inhibited by the PKC inhibitors wortmannin, GF 109203X, and Gö 6983. Considering the marked inhibitory effect of wortmannin on PKC ι and evidence that the maximum stimulation of PI3K was observed at 5 min after the addition of G-CSF, PI3K was determined to be the upstream regulator of PKC ι in the G-CSF signal transduction of HL-60 cells. The basal activity of PKC ι in Trf-R $^{+}$ cells was higher than that in Trf-R $^{-}$ cells, and G-CSF was more augmented. In Trf-R $^{-}$ cells, PKC ι activity was scarcely stimulated by G-CSF. This tendency of PKC ι to be activated by G-CSF was similar to that of PI3K (Kanayasu-Toyoda et al., 2002).

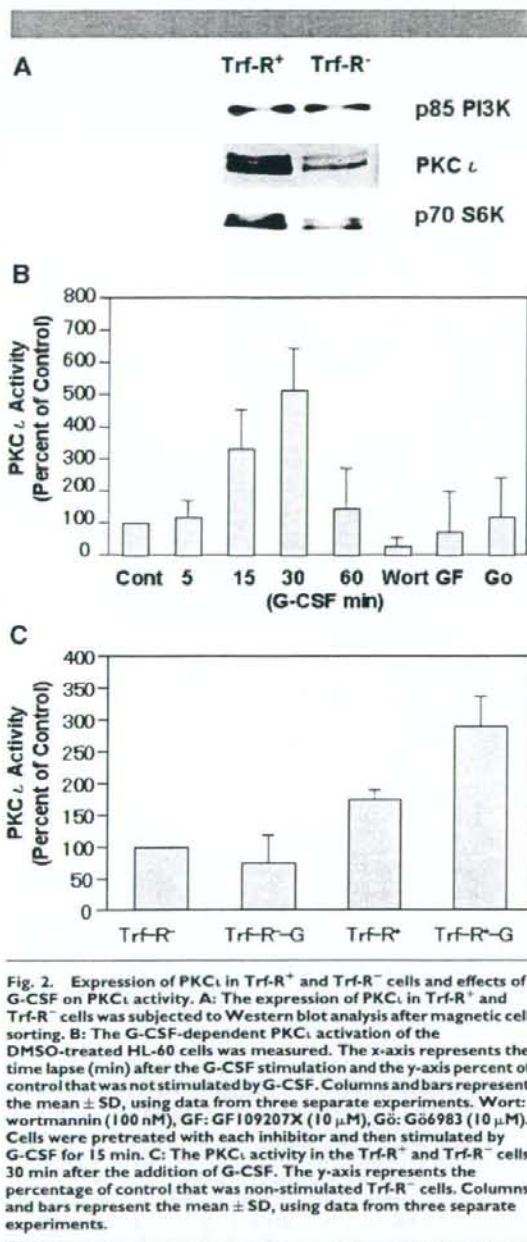


Fig. 2. Expression of PKC ζ in Trf-R⁺ and Trf-R⁻ cells and effects of G-CSF on PKC ζ activity. **A:** The expression of PKC ζ in Trf-R⁺ and Trf-R⁻ cells was subjected to Western blot analysis after magnetic cell sorting. **B:** The G-CSF-dependent PKC ζ activation of the DMSO-treated HL-60 cells was measured. The x-axis represents the time lapse (min) after the G-CSF stimulation and the y-axis percent of control that was not stimulated by G-CSF. Columns and bars represent the mean \pm SD, using data from three separate experiments. Wort: wortmannin (100 nM), GF: GF109207X (10 μ M), Go: Gö6983 (10 μ M). Cells were pretreated with each inhibitor and then stimulated by G-CSF for 15 min. **C:** The PKC ζ activity in the Trf-R⁺ and Trf-R⁻ cells 30 min after the addition of G-CSF. The y-axis represents the percentage of control that was non-stimulated Trf-R⁻ cells. Columns and bars represent the mean \pm SD, using data from three separate experiments.

Effects of G-CSF on PKC ζ translocation

Muscella et al. (2003) demonstrated that the translocation of PKC ζ from the cytosol to the nucleus or membrane is required for c-Fos synthesis induced by angiotensin II in MCF-7 cells. It was also reported that high glucose induced the translocation of PKC ζ (Chuang et al., 2003). These results suggest that the translocation of aPKC plays an important role in its signaling. To clarify the translocation of PKC ζ , immuno-histochemical staining (Fig. 3) and biochemical fractionation (Fig. 4) in

DMSO-induced HL-60 cells were performed after the addition of G-CSF. In a non-stimulated condition, PKC ζ in the HL-60 cells treated with DMSO for 2 days (Fig. 3, control) was detected mainly in the nucleus. Analysis of Western blotting (Fig. 4, left parts) and quantification of the bands (Fig. 4, right columns) also revealed that PKC ζ was localized and observed mainly in the nuclear fraction (Fig. 4A). During the 5–15 min period after the addition of G-CSF, PKC ζ was found to translocate (Figs. 3 and 4B) into the membrane fraction, after which it re-translocated into the cytosol fraction (Fig. 4C). In the presence of wortmannin, the G-CSF-induced translocation of PKC ζ into the plasma membrane failed, but PKC ζ was found to localize in the cytosolic fraction (Figs. 3 and 4B). Myeloperoxidase is thought to be expressed in stage from promyelocytes to mature neutrophils (Manz et al., 2002). In human cord blood cells (Fig. 3), PKC ζ in the cells co-stained with anti-myeloperoxidase antibody was also localized in the nucleus after serum depletion (Fig. 3B top parts). Ten minutes after the addition of G-CSF, PKC ζ was found to translocate into the membrane, and then into the cytosol at 30 min after the addition of G-CSF. In the presence of wortmannin, the G-CSF-induced translocation of PKC ζ into the plasma membrane failed but PKC ζ was found to localize in the cytosol. This suggested that the dynamic translocation of PKC ζ induced by G-CSF is a universal phenomenon in neutrophilic lineage cells. Taken together, these data support the possibility that PI3K plays not only an important role upstream of PKC ζ but also triggers the translocation from nucleus to membrane upon the addition of G-CSF.

In order to assess the purity of each cellular fraction, antibodies against specific markers were blotted. As specific markers, Histon-H1, Fc γ receptor IIa (CD32), and lactate dehydrogenase (LDH) were used for the nuclear, membrane, and cytosolic fractions, respectively. The purities of the nuclear, membrane, and cytosolic fractions were 82.0, 78.5, and 72.2%, respectively (Fig. 4D).

Effects of siRNA for PKC ζ on proliferation and differentiation

To determine the role of PKC ζ in neutrophilic proliferation and differentiation, PKC ζ was knocked down by siRNA. When the protein level of PKC ζ was specifically downregulated by siRNA for PKC ζ (Fig. 5A), G-CSF failed to enhance proliferation of the cells during 5 days' cultivation (Fig. 5B). The effect of siRNA for PKC ζ on neutrophilic differentiation in terms of fMLP-R expression was also determined. As shown in Figure 5C, fMLP-R expression was promoted by siRNA for PKC ζ in either the presence (lower part) or absence (upper part) of G-CSF. These data indicate that PKC ζ positively regulates G-CSF-induced proliferation and negatively regulates the differentiation of DMSO-treated HL-60 cells.

Discussion

We previously reported that PI3K/p70 S6K plays an important role in the regulation of the neutrophilic differentiation and proliferation of HL-60 cells. Akimoto et al. (1998) and Romanelli et al. (1999) reported that p70 S6K is regulated by aPKC and aPKC λ /PKC ζ , respectively. At first, we showed that the distribution of PKC ζ and PKC λ proteins in various human tissues and cells was not similar (Fig. 1A), and that PKC ζ are more abundantly expressed in proliferating blood cells: Jurkat, K562, U937, and HL-60 cells (Fig. 1B). Moreover, PKC ζ proteins were also observed in cultured mononuclear cells of cord blood, in which the myeloid progenitors were enriched in the presence or absence of G-CSF (Fig. 1B). The myeloperoxidase-positive cells as neutrophilic lineage cells, a myeloid marker, were also stained with the antibody of PKC ζ (Fig. 3B). Although PKC ζ proteins are barely detected in

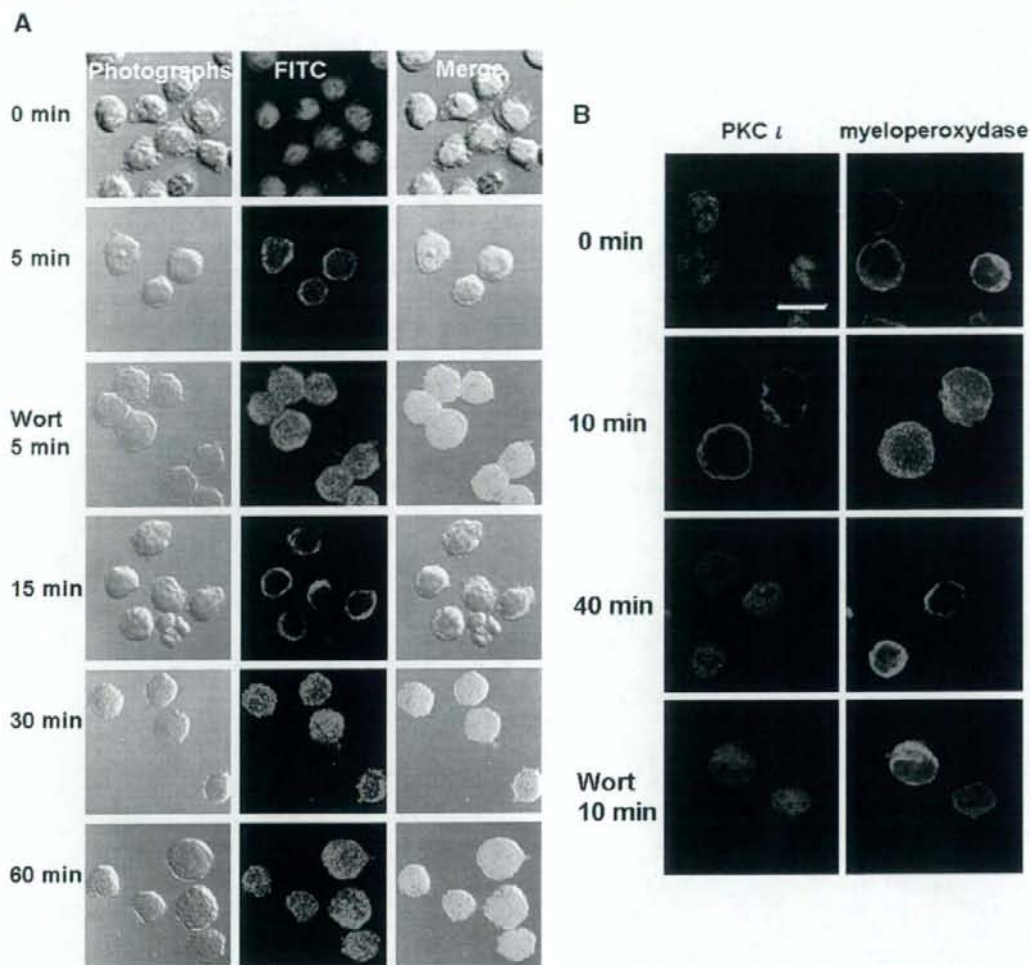


Fig. 3. Translocation of PKC ζ after the activation of G-CSF. A: 2 days after the addition of DMSO, HL-60 cells stimulated by G-CSF were fixed, incubated with anti-PKC ζ antibody, and visualized as described above. The photographs can be seen at the left part of the figure, the fluorescent photographs in the middle of the figure, and the merged images at the right. B: G-CSF-stimulated mononuclear cells from cord blood were stained with anti-PKC ζ antibody (red, left part) and anti-myeloperoxidase antibody (green, right part) after serum depletion. Under no stimulation, PKC ζ was observed in the nucleus. G-CSF promoted the translocation of PKC ζ to the membrane within 5–15 min, and then to the cytosol. Wort: wortmannin. White bar: 10 μ m.

neutrophilic HL-60 cells, PKC ζ proteins were markedly expressed in these cells (Fig. 1B). This study showed, for the first time, the stimulation of PKC ζ activity in G-CSF-treated HL-60 cells (Fig. 2B) at 15–30 min after the addition of G-CSF. Maximum activation from the addition of NGF in PC12 cells was also observed at 15 min (Wooten et al., 2001). Atypical PKCs are lipid-regulated kinases that need to be localized to the membrane in order to be activated. PKC ζ is directly activated by phosphatidylinositol 3,4,5-trisphosphate, a product of PI3K (Nakanishi et al., 1993). We previously reported that the maximum activation of PI3K was observed in HL-60 cells 5 min after the addition of G-CSF (Kanayasu-Toyoda et al., 2002). Most investigators have reported the translocation of aPKC in either muscle cells or adipocytes stimulated by insulin (Andjelkovic et al., 1997; Goransson et al.,

1998; Galetic et al., 1999; Standaert et al., 1999; Braiman et al., 2001; Chen et al., 2003; Kanzaki et al., 2004; Sasaoka et al., 2004; Herr et al., 2005). In response to insulin stimulation, aPKC ζ/λ is translocated to the plasma membrane (Standaert et al., 1999; Braiman et al., 2001), where aPKC ζ/λ is believed to be activated (Galetic et al., 1999; Kanzaki et al., 2004). In the present study, the addition of G-CSF induced PKC ζ to translocate to the membrane from the nucleus within 5–15 min (Figs. 3 and 4), and this translocation to the plasma membrane accompanied the full activation of PKC ζ (Fig. 2B). Previously we reported also that the maximum activation of p70 S6K in HL-60 cells was observed from 30 to 60 min after the addition of G-CSF (Kanayasu-Toyoda et al., 1999, 2002), suggesting that there was a time lag between the activation of PI3K and p70 S6K upon the addition of G-CSF in HL-60 cells. In the present study, PKC ζ was

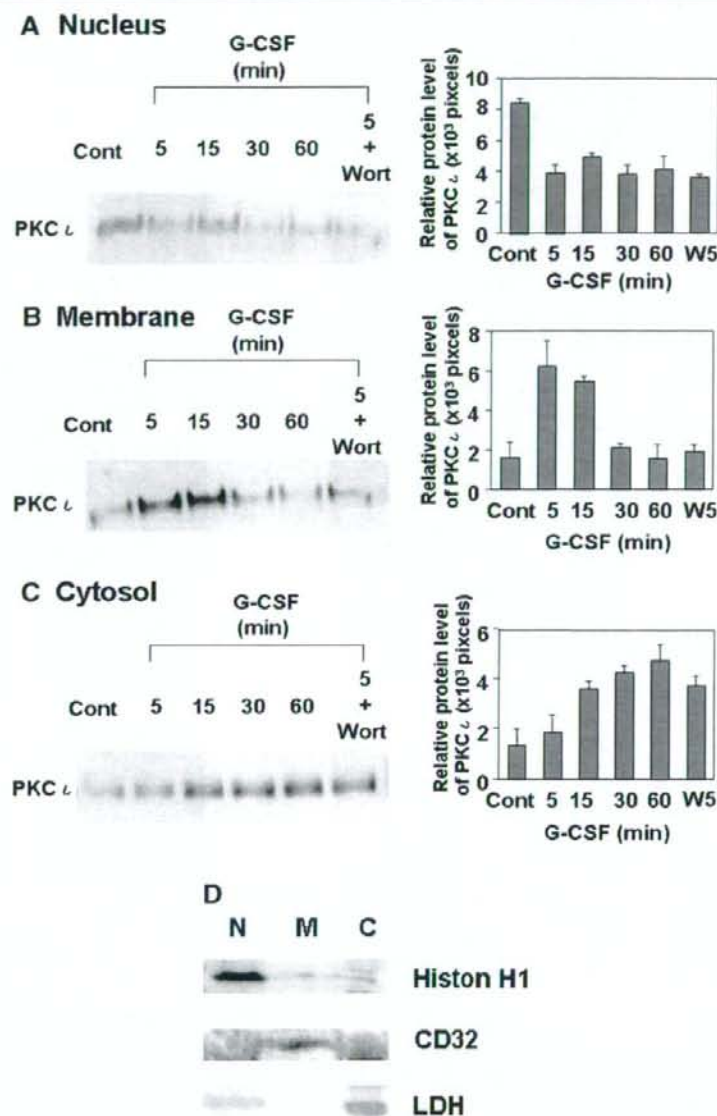


Fig. 4. Translocation of PKC ι after activation by G-CSF on biochemical fractionation. The cells were differentiated as described in the Figure 3 legend. After stimulation by G-CSF, the amounts of PKC ι proteins in the nucleus (A), plasma membrane (B), and cytosol (C), as fractionated by differential centrifugation, were analyzed by Western blotting (left parts). The right parts show the quantitation of the bands of PKC ι proteins. Wort or W: wortmannin. PKC ι protein was quantitated using data from three separate experiments. Columns and bars represent the mean \pm SD. D: Each cell fraction was immunoblotted with antibodies of specific marker. Histon-H1, Fc γ receptor IIa (CD32), and lactate dehydrogenase (LDH) are specific markers for nuclear (N), membrane (M), and cytosolic (C) fractions, respectively.

found to re-translocate from the plasma membrane to the cytosol (Figs. 3 and 4C). In the presence of wortmannin, an inhibitor of PI3K, PKC ι failed to translocate into the plasma membrane, but instead translocated to cytosol directly from the nucleus upon the addition of G-CSF (Figs. 3 and 4B). PKC ι translocation was also observed in myeloperoxidase-positive cells derived from human cord blood (Fig. 3B), indicating that G-CSF-induced dynamic translocation of PKC ι occurred in not

only a limited cell line but also neutrophilic lineage cells. These data suggest that PI3K plays an important role in the activation and translocation of PKC ι during the G-CSF-induced activation of myeloid cells. Furthermore, the translocation to the plasma membrane in response to G-CSF is wortmannin sensitive, but the translocation from the nucleus upon G-CSF stimulation is not affected by wortmannin, suggesting that the initial signal of G-CSF-induced PKC ι translocation from the nucleus may be

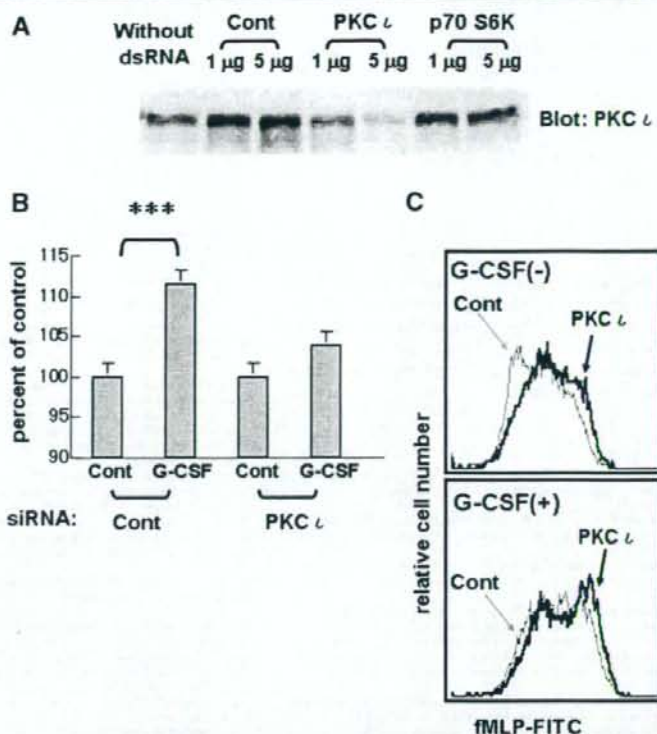


Fig. 5. Effects of siRNA of PKC ζ on proliferation, differentiation, and phosphorylation at various sites of p70 S6K. **A:** Forty-eight hours after transfection with siRNA of PKC ζ or p70 S6K, protein levels of PKC ζ were compared. **B:** Proliferation of the cells transfected with siRNA of PKC ζ or control (Cont) was measured 5 days after the addition of G-CSF. Columns and bars represent the mean \pm SD of triplicate wells (** P < 0.001). **C:** fMLP-R expression was analyzed by flow cytometry 5 days after the addition of G-CSF. The gray arrow indicates cells transfected with the control sequence of double-stranded RNA (Cont, gray lines), and the black arrow the cells transfected with siRNA for PKC ζ (black lines) in the presence (lower part) or absence (upper part) of G-CSF.

PI3K-independent, but association of PKC ζ with the plasma membrane could be mediated through a PI3K-dependent signal. Cord blood is an important material of blood transplantation for leukemia (Bradstock et al., 2006; Ooi, 2006; Yamada et al., 2006) or for congenital neutropenia (Mino et al., 2004; Nakazawa et al., 2004) because it contains many hematopoietic stem cells such as CD34-positive cells or CD133-positive cells, and also contains immature granulocytes. The neutrophilic differentiation and proliferation are necessary processes after transplantation.

Formyl-Met-Leu-Phe peptide evokes the migration, superoxide production, and phagocytosis of neutrophils through fMLP-R, a suitable marker for neutrophilic differentiation. In this study, the reduction of PKC ζ by siRNA inhibited G-CSF-induced proliferation (Fig. 5B) and promoted neutrophilic differentiation (Fig. 5C) in terms of fMLP-R expression. These data, however, suggest that PKC ζ promoted G-CSF-induced proliferation and blocked differentiation at the same time. The substrates of aPKC have recently been reported; namely, the cytoskeletal protein Lethal giant larvae (Lgl) was phosphorylated by Drosophila aPKC (Betschinger et al., 2003) and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was phosphorylated by PKC ζ (Tisdale, 2002) directly in both cases. While the direct phosphorylation of p70 S6K by aPKC was not observed (Akimoto et al., 1998; Romanelli et al.,

1999), the enzyme activity of p70 S6K was markedly enhanced by co-transfection with aPKC and PDK-1, the latter of which is recruited to the membrane due to the binding of phosphatidylinositol-3,4,5-trisphosphate to its PH domain (Anderson et al., 1998). The addition of G-CSF induced PKC ζ to increase phosphorylation at Thr-389, which is the site most closely related to enzyme activity among the multi-phosphorylation sites of p70 S6K (Weng et al., 1998). However, the mammalian target of rapamycin (mTOR), an upstream regulator, also phosphorylates Thr-389 of p70 S6K and markedly stimulates p70 S6K activity under coexistence with PDK-1 (Isotani et al., 1999). We could not rule out the possibility that other PKC isoforms can contribute to the activation of p70 S6K. We postulated that in G-CSF-stimulated HL-60 cells, PKC ζ contributes to p70 S6K activation as an upstream regulator.

Atypical PKC isoforms are reported to play an important role in the activation of I κ B kinase β (Lallena et al., 1999). In PKC ζ -deficient mice, impaired signaling through the B-cell receptor resulted in the inhibition of cell proliferation and survival while also causing defects in the activation of ERK and the transcription of NF- κ B-dependent genes (Martin et al., 2002). Moreover, Lafuente et al. (2003) demonstrated that the loss of Par-4, that is, the genetic inactivation of the aPKC inhibitor, led to an increased proliferative response of

peripheral T cells when challenged through the T-cell receptor. However, it has been reported that PKC λ -deficient mice have a lethal phenotype at the early embryonic stage (Soloff et al., 2004). Based on the present results and those of previous reports (Kanayasu-Toyoda et al., 1999, 2002), we postulate that PKC λ plays an important role in regulating G-CSF-induced proliferation in neutrophilic lineage cells.

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Literature Cited

Akimoto K, Nakaya M, Yamana T, Tanaka J, Matsuda S, Weng QP, Avruch J, Ohno S. 1998. Atypical protein kinase C λ binds and regulates p70 S6 kinase. *Biochem J* 335:417-424.

Anderson KE, Coadwell J, Stephens LR, Hawkins PT. 1998. Translocation of PDK-1 to the plasma membrane is important in allowing PDK-1 to activate protein kinase B. *Curr Biol* 8:684-691.

Andjelkovic M, Alessi DR, Meier R, Fernandez A, Lamb NJ, Frech M, Cron P, Cohen P, Lucocq JM, Hemmings BA. 1997. Role of translocation in the activation and function of protein kinase B. *J Biol Chem* 272:31515-31524.

Betschinger J, Mechler K, Knoblich JA. 2003. The Par complex directs asymmetric cell division by phosphorylating the cytoskeletal protein Lgl. *Nature* 423:326-330.

Bradstock KF, Hertzberg MS, Kerridge JH, Svernlind J, McGurgan M, Huang G, Antonenas V, Gottlieb DJ. 2006. Unrelated umbilical cord blood transplantation for adults with haematological malignancies: Results from a single Australian centre. *Intern Med J* 36:355-361.

Braiman L, Alt A, Kuraki T, Ohba M, Bak A, Tenenbaum T, Sampson SR. 2001. Activation of protein kinase C zeta induces serine phosphorylation of VAMP2 in the GLUT4 compartment and increases glucose transport in skeletal muscle. *Mol Cell Biol* 21:7852-7861.

Chen X, Al-Hasani H, Olausson T, Wenzel AM, Smith U, Cushman SW. 2003. Activity, phosphorylation state and subcellular distribution of GLUT4-targeted Akt2 in rat adipose cells. *J Cell Sci* 116:3511-3518.

Chuang LY, Guh JY, Liu SF, Hung MY, Liao TN, Chiang TA, Huang JS, Huang YL, Lin CF, Yang YL. 2003. Regulation of type II transforming-growth-factor-beta receptors by protein kinase C ι . *Biochem J* 375:385-393.

Chung J, Grammer TC, Lemon KP, Kazaluskas A, Blenis J. 1994. PDGF- and insulin-dependent p70 S6K activation mediated by phosphatidylinositol-3-OH kinase. *Nature* 370:71-75.

Debill N, Robin C, Schiavon V, Letestu R, Pluimio F, Mitjavila-Garcia MT, Coulombel L, Vainchenker W. 2001. Different expression of CD41 on human lymphoid and myeloid progenitors from adults and neonates. *Blood* 97:2023-2030.

Fritsch G, Buchinger P, Printz D, Fink FM, Mann G, Peters C, Wagner T, Adler A, Gadner H. 1993. Rapid discrimination of early CD34⁺ myeloid progenitors using CD45-RA analysis. *Blood* 81:2301-2309.

Galetic I, Andjelkovic M, Meier R, Brodbeck D, Park J, Hemmings BA. 1999. Mechanism of protein kinase B activation by insulin/insulin-like growth factor-1 revealed by specific inhibitors of phosphoinositide 3-kinase—Significance for diabetes and cancer. *Pharmacol Ther* 82:409-425.

Goransson O, Wijkander J, Manganiello V, Degerman E. 1998. Insulin-induced translocation of protein kinase B to the plasma membrane in rat adipocytes. *Biochem Biophys Res Commun* 246:249-254.

Hao QL, Zhu J, Price MA, Payne KJ, Barsky LW, Crooks GM. 2001. Identification of a novel, human myeloid progenitor in cord blood. *Blood* 97:3683-3690.

Herr HJ, Bernard JR, Reeder DW, Rivas DA, Limon JJ, Yaspelkis BB3rd. 2005. Insulin-stimulated plasma membrane association and activation of Akt2, aPKC zeta and aPKC lambda in high fat fed rodent skeletal muscle. *J Physiol* 565:627-636.

Huang S, Chen Z, Yu JF, Young D, Bashey A, Ho AD, Law P. 1999. Correlation between IL-3 receptor expression and growth potential of human CD34⁺ hematopoietic cells from different tissues. *Stem Cells* 17:265-272.

Isotani S, Hara K, Tokunaga C, Inoue H, Avruch J, Yonezawa K. 1999. Immunopurified mammalian target of rapamycin phosphorylates and activates p70 S6 kinase α in vitro. *J Biol Chem* 274:34493-34498.

Kanayasu-Toyoda T, Yamaguchi T, Uchida E, Hayakawa T. 1999. Commitment of neutrophilic differentiation and proliferation of HL-60 cells coincides with expression of transferrin receptor. Effect of granulocyte colony stimulating factor on differentiation and proliferation. *J Biol Chem* 274:25471-25480.

Kanayasu-Toyoda T, Yamaguchi T, Oshizawa T, Kogi M, Uchida E, Hayakawa T. 2002. Role of the p70 S6 kinase cascade in neutrophilic differentiation and proliferation of HL-60 cells—a study of transferrin receptor-positive and -negative cells obtained from dimethyl sulfoxide- or retinoic acid-treated HL-60 cells. *Arch Biochem Biophys* 405:21-31.

Kanayasu-Toyoda T, Yamaguchi T, Oshizawa T, Uchida E, Hayakawa T. 2003. The role of c-Myb on granulocyte colony-stimulating factor-dependent neutrophilic proliferation and differentiation of HL-60 cells. *Biochem Pharmacol* 66:133-140.

Kanzaki M, Mora S, Hwang JB, Sattiel AR, Pesin JE. 2004. Atypical protein kinase C (PKC ζ /lambda) is a convergent downstream target of the insulin-stimulated phosphatidylinositol 3-kinase and TC10 signaling pathways. *J Cell Biol* 164:279-290.

Lafuente MJ, Martin P, Garcia-Cao I, Diaz-Meco MT, Serrano M, Mosaic J. 2003. Regulation of mature T lymphocyte proliferation and differentiation by Par-4. *Embo J* 22:4689-4698.

Lallena MJ, Diaz-Meco MT, Bren G, Pava CV, Mosaic J. 1999. Activation of IkappaB kinase beta by protein kinase C isoforms. *Mol Cell Biol* 19:2180-2188.

Liu WS, Heckman CA. 1998. The sevenfold way of PKC regulation. *Cell Signal* 10:529-542.

Marz MG, Miyamoto T, Akashi K, Weissman IL. 2002. Prospective isolation of human clonogenic common myeloid progenitors. *Proc Natl Acad Sci USA* 99:11872-11877.

Martin P, Duran A, Minguez S, Gaspar ML, Diaz-Meco MT, Rennett P, Leitges M, Mosaic J. 2002. Role of zeta PKC in B-cell signaling and function. *Embo J* 21:4049-4057.

Mino E, Kobayashi R, Yoshida M, Suzuki Y, Yamada M, Kobayashi K. 2004. Umbilical cord blood stem cell transplantation from unrelated HLA-matched donor in an infant with severe congenital neutropenia. *Bone Marrow Transplant* 33:969-971.

Muscella A, Greco S, Elia MG, Storelli C, Marsigliante S. 2003. PKC- ζ is required for angiotensin II-induced activation of ERK and synthesis of C-FOS in MCF-7 cells. *J Cell Physiol* 197:61-68.

Nakanishi H, Brewer KA, Exton JH. 1993. Activation of the zeta isozyme of protein kinase C by phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem* 268:13-16.

Nakazawa Y, Sakashita K, Kinoshita M, Saida K, Shigemura T, Yanagisawa R, Shikama N, Kamijo T, Koike K. 2004. Successful unrelated cord blood transplantation using a reduced-intensity conditioning regimen in a 6-month-old infant with congenital neutropenia complicated by severe pneumonia. *Int J Hematol* 80:287-290.

Nguyen BT, Dessauer CW. 2005. Relaxin stimulates protein kinase C zeta translocation: Requirement for cyclic adenosine 3',5'-monophosphate production. *Mol Endocrinol* 19:1012-1023.

Ooi J. 2006. The efficacy of unrelated cord blood transplantation for adult myelodysplastic syndrome. *Leuk Lymphoma* 47:599-602.

Rappold I, Ziegler BL, Kohler I, Marchetto S, Rosnet O, Birnbaum D, Simmons PJ, Zannetto AC, Hill B, Neu S, Knapp W, Altiro R, Altiro K, Ullrich A, Kanz L, Buhning HJ. 1997. Functional and phenotypic characterization of cord blood and bone marrow subsets expressing FLT3 (CD135) receptor tyrosine kinase. *Blood* 90:111-125.

Romanelli A, Martin KA, Tokar A, Blenis J. 1999. p70 S6 kinase is regulated by protein kinase C ζ and participates in a phosphoinositide 3-kinase-regulated signaling complex. *Mol Cell Biol* 19:2921-2928.

Sasaka T, Wada T, Fukui K, Murakami S, Ishihara H, Suzuki R, Tobe K, Kadawaki T, Kobayashi M. 2004. SH2-containing inositol phosphatase 2 predominantly regulates Akt2, and not Akt1, phosphorylation at the plasma membrane in response to insulin in 3T3-L1 adipocytes. *J Biol Chem* 279:14835-14843.

Seibler LA, Schmitz-Peiffer C, Sheng Y, Biden TJ. 1993. Molecular cloning and characterization of PKC ι , an atypical isoform of protein kinase C derived from insulin-secreting cells. *J Biol Chem* 268:24296-24302.

Soloff RS, Katayama C, Lin MY, Feramisco JR, Hedrick SM. 2004. Targeted deletion of protein kinase C lambda reveals a distribution of functions between the two atypical protein kinase C isoforms. *J Immunol* 173:3250-3260.

Standaert ML, Bandopadhyay G, Perez L, Price D, Galloway L, Poklepovic A, Sajan MP, Cenni V, Sri A, Mosaic J, Tokar A, Farese RV. 1999. Insulin activates protein kinases C-zeta and C-lambda by an autophosphorylation-dependent mechanism and stimulates their translocation to GLUT4 vesicles and other membrane fractions in rat adipocytes. *J Biol Chem* 274:25308-25316.

Tenen DG, Hromas R, Licht JD, Zhang DE. 1997. Transcription factors, normal myeloid development, and leukemia. *Blood* 90:489-519.

Tisdale EJ. 2002. Glyceraldehyde-3-phosphate dehydrogenase is phosphorylated by protein kinase C ι /lambda and plays a role in microtubule dynamics in the early secretory pathway. *J Biol Chem* 277:3334-3341.

Ward AC, Loeb DM, Soete-Bobok AA, Tow IP, Friedman AD. 2000. Regulation of granulopoiesis by transcription factors and cytokine signals. *Leukemia* 14:973-990.

Weng QP, Kozlowski M, Belham C, Zhang A, Comb MJ, Avruch J. 1998. Regulation of the p70 S6 kinase by phosphorylation in vivo. Analysis using site-specific anti-phosphopeptide antibodies. *J Biol Chem* 273:16621-16629.

Wooten MW, Vandemulst ML, Seibler ML, Geetha T, Diaz-Meco MT. 2001. Nerve growth factor stimulates mitotic tyrosine phosphorylation and activation of the atypical protein kinase C ζ via a src kinase pathway. *Mol Cell Biol* 21:8414-8427.

Yamada K, Mizusawa M, Harima A, Kajiwara K, Haraki T, Hoshi K, Kozai Y, Kodo H. 2006. Induction of remission of relapsed acute myeloid leukemia after unrelated donor cord blood transplantation by concomitant low-dose cytarabine and calcitriol in adults. *Eur J Haematol* 77:345-348.

Yamaguchi T, Mukasa T, Uchida E, Kanayasu-Toyoda T, Hayakawa T. 1999. The role of STAT3 in granulocyte colony-stimulating factor-induced enhancement of neutrophilic differentiation of Me2SO-treated HL-60 cells. GM-CSF inhibits the nuclear translocation of tyrosine-phosphorylated STAT3. *J Biol Chem* 274:15575-15581.



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.

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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 cases 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% CO₂ 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 H₂O 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 through a solid-phase extraction cartridge (SepPak C-18, Waters, Milford, MA, USA) with 2 ml of H₂O, dried under vacuum, and resolved in 50 µl of H₂O. 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-

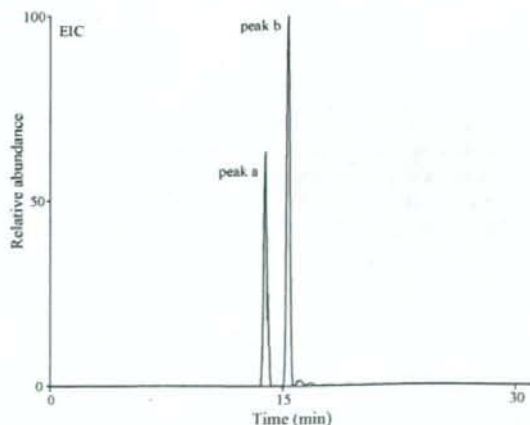


Fig. 1. EIC at m/z 426.13–426.17 and m/z 442.12–442.16 obtained by SIM (m/z 400–450) of DMB-NeuGc and DMB-NeuAc in the positive ion mode.

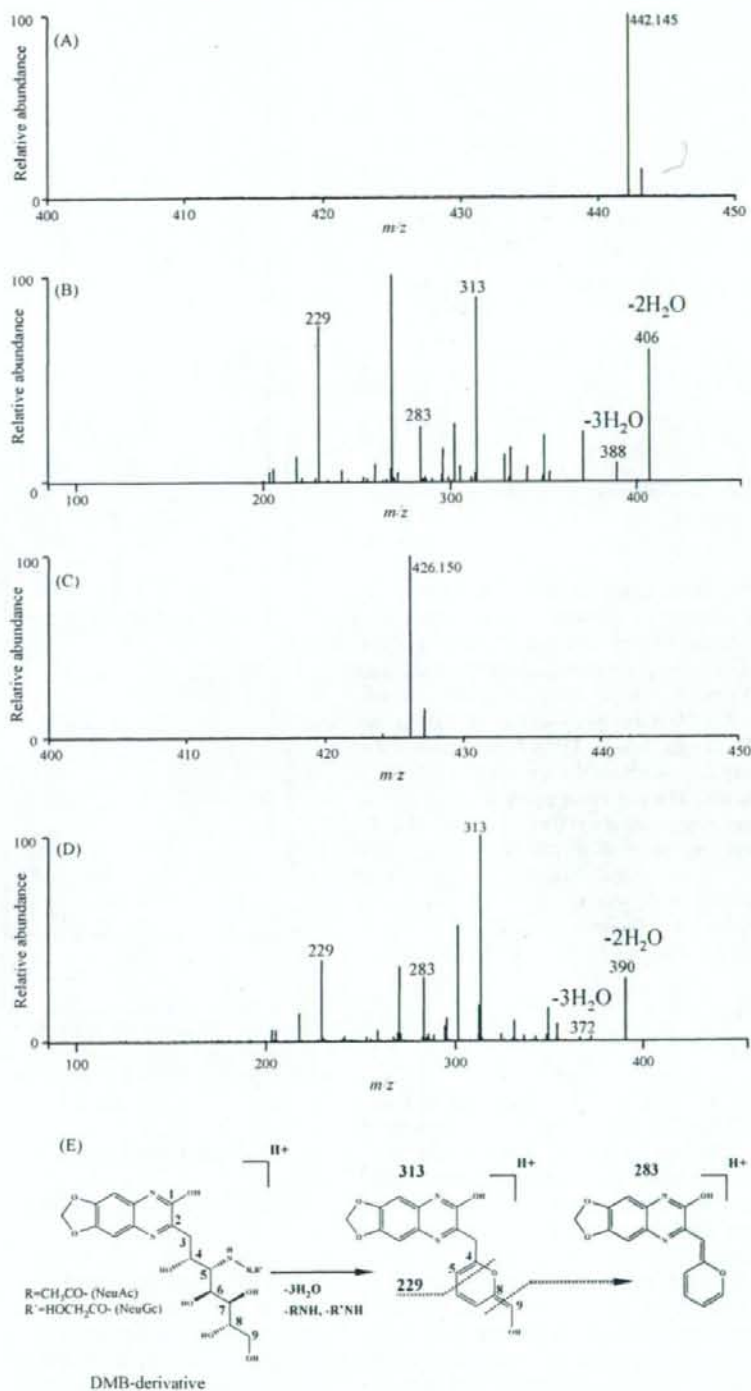


Fig. 2. (A) Typical MS spectrum of peak a. (B) MS/MS spectrum of $[M+H]^+$ (m/z 442.145) acquired from around peak a. (C) Typical MS spectrum of peak b. (D) MS/MS spectrum of $[M+H]^+$ (m/z 426.150) acquired from around peak b. (E) Fragmentation of DMB-NeuGe and DMB-NeuAc.

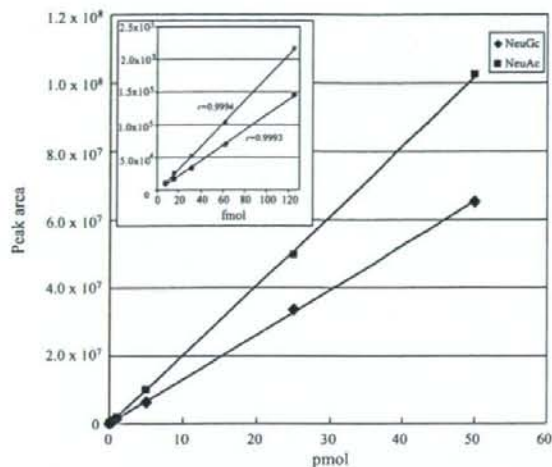


Fig. 3. Calibration curves of DMB-NeuGc ($r=0.9998$) and DMB-NeuAc ($r=0.9995$).

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 a 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.

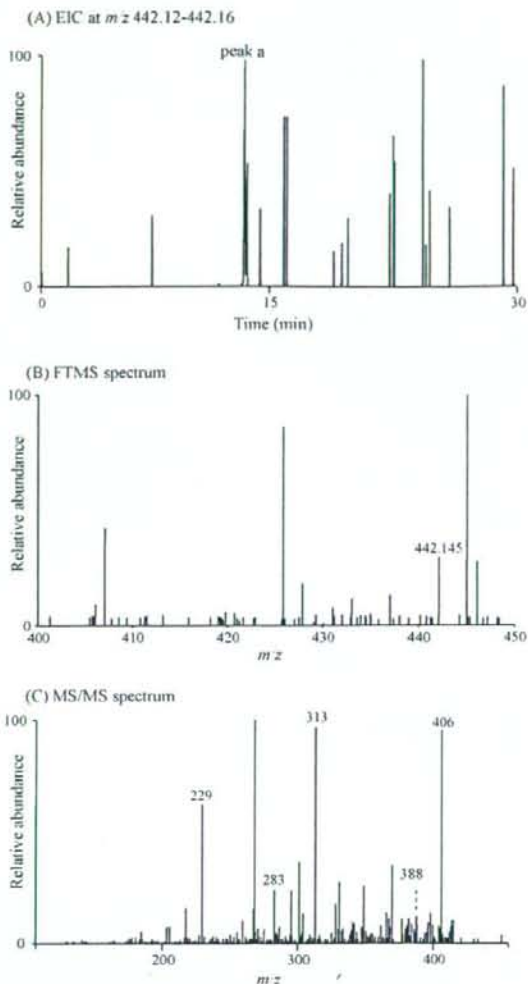


Fig. 4. Detection of DMB-NeuGc in the membrane fractions of HL-60RG cells (2.5×10^3) cultured with 10% FCS. (A) EIC at m/z 442.12–442.16 obtained by SIM. (B) Typical MS spectrum of peak a. (C) MS/MS spectrum of $[M+H]^+$ (m/z 442.145) acquired from around peak a.

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-

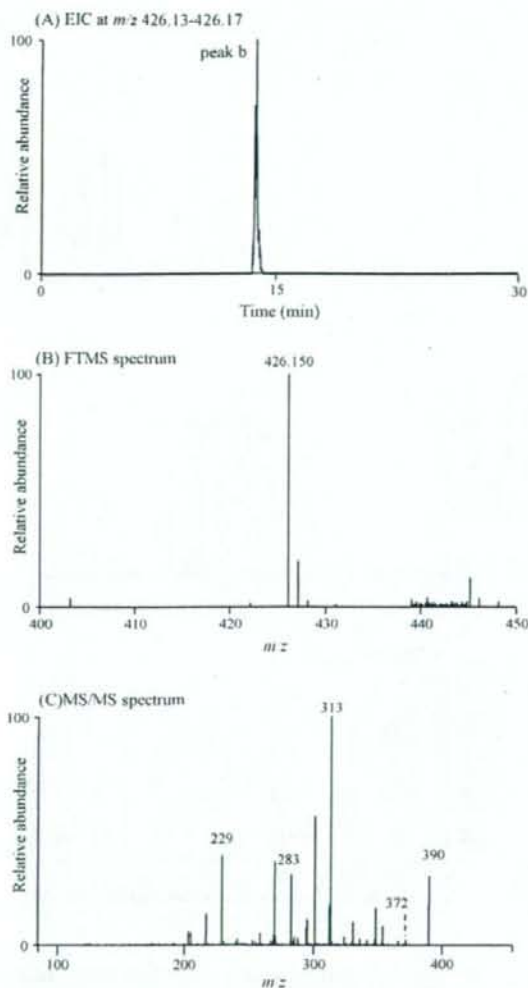


Fig. 5. Detection of DMB-NeuAc in the membrane fractions of HL-60RG cells (2.5×10^3) cultured with 10% FCS. (A) EIC at m/z 426.13–426.17 obtained by SIM. (B) Typical MS spectrum of peak b. (C) MS/MS spectrum of $[M+H]^+$ (m/z 426.150) acquired from around peak b.

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

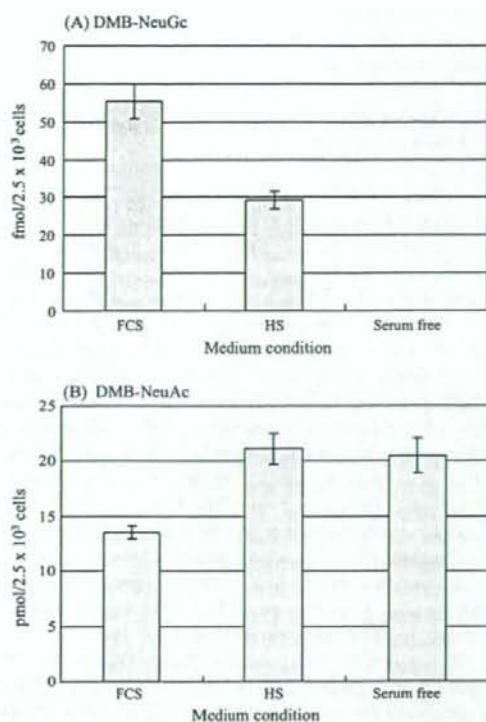


Fig. 6. Levels of (A) NeuGc and (B) NeuAc in the membrane fraction of HL-60RG cells (2.5×10^3) cultured with 10% FCS, 10% human serum (HS) and serum-free medium. Values are the means \pm SD ($n=3$).

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

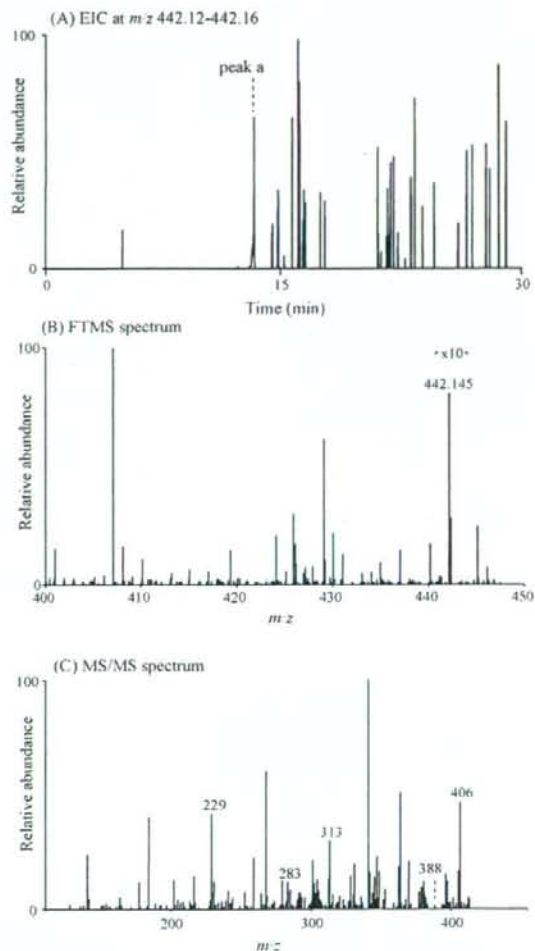


Fig. 7. Detection of DMB-NeuGc in the membrane fractions of HL-60RG cells (2.5×10^3) cultured with 10% human serum. (A) EIC at m/z 442.12–442.16 obtained by SIM. (B) Typical MS spectrum of peak a. (C) MS/MS spectrum of $[M+H]^+$ (m/z 442.145) acquired from around peak a.

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.

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References

- [1] C. Traving, R. Schauer, *Cell Mol. Life Sci.* 54 (1998) 1330.
- [2] T. Angata, A. Varki, *Chem. Rev.* 102 (2002) 439.
- [3] A. Varki, *Glycobiology* 2 (1992) 25.
- [4] R. Schauer, *Adv. Carbohydr. Chem. Biochem.* 40 (1982) 131.
- [5] S. Kitazume, K. Kitajima, S. Inoue, S.M. Haslam, H.R. Morris, A. Dell, W.J. Lennarz, Y. Inoue, *J. Biol. Chem.* 271 (1996) 6694.
- [6] R. Schauer, J. Haverkamp, M. Wember, J.P. Kamerling, J.F. Vliegenthart, *Eur. J. Biochem.* 62 (1976) 237.
- [7] N. Kawasaki, S. Itoh, M. Ohta, T. Hayakawa, *Anal. Biochem.* 316 (2003) 15.
- [8] M. Nakano, K. Kakehi, M.H. Tsai, Y.C. Lee, *Glycobiology* 14 (2004) 431.
- [9] E.A. Muchmore, S. Diaz, A. Varki, *Am. J. Phys. Anthropol.* 107 (1998) 187.
- [10] A. Irie, S. Koyama, Y. Kozutsumi, T. Kawasaki, A. Suzuki, *J. Biol. Chem.* 273 (1998) 15866.
- [11] H.H. Chou, H. Takematsu, S. Diaz, J. Iber, E. Nickerson, K.L. Wright, E.A. Muchmore, D.L. Nelson, S.T. Warren, A. Varki, *Proc. Natl. Acad. Sci. U. S. A.* 95 (1998) 11751.
- [12] H. Higashi, M. Naiki, S. Matuo, K. Okouchi, *Biochem. Biophys. Res. Commun.* 79 (1977) 388.
- [13] J.M. Merrick, K. Zadarlik, F. Milgrom, *Int. Arch. Allergy Appl. Immunol.* 57 (1978) 477.
- [14] M.J. Martin, A. Muotri, F. Gage, A. Varki, *Nat. Med.* 11 (2005) 228.
- [15] A. Heiskanen, T. Satomaa, S. Tiitinen, A. Laitinen, S. Mannelin, U. Impola, M. Mikkola, C. Olsson, H. Miller-Podraza, M. Blomqvist, A. Olonen, H. Salo, P. Lehenkari, T. Tuuri, T. Otonkoski, J. Natunen, J. Saarinen, J. Laine, *Stem Cells* 25 (2007) 197.
- [16] A.E. Manzi, S. Diaz, A. Varki, *Anal. Biochem.* 188 (1990) 20.
- [17] S. Hara, M. Yamaguchi, Y. Takemori, K. Furuhashi, H. Ogura, M. Nakamura, *Anal. Biochem.* 179 (1989) 162.
- [18] M. Bardor, D.H. Nguyen, S. Diaz, A. Varki, *J. Biol. Chem.* 280 (2005) 4228.
- [19] M. Ito, K. Ikeda, Y. Suzuki, K. Tanaka, M. Saito, *Anal. Biochem.* 300 (2002) 260.
- [20] F.N. Lamari, N.K. Karamanos, *J. Chromatogr. B* 781 (2002) 3.
- [21] A. Klein, S. Diaz, I. Ferreira, G. Lamblin, P. Roussel, A.E. Manzi, *Glycobiology* 7 (1997) 421.
- [22] H.H. Chou, T. Hayakawa, S. Diaz, M. Krings, E. Indriati, M. Leakey, S. Paabo, Y. Satta, N. Takahata, A. Varki, *Proc. Natl. Acad. Sci. U. S. A.* 99 (2002) 11736.
- [23] P. Tangvoranuntakul, P. Gagneux, S. Diaz, M. Bardor, N. Varki, A. Varki, E. Muchmore, *Proc. Natl. Acad. Sci. U. S. A.* 100 (2003) 12045.

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) proof 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 rationale 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 (EMA) 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 rationale 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 constraining 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, tumorigenicity, 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) I 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]:

Selectivity =

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

"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 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
EMA = 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

REFERENCES

- Southam, C. M.; Moore, A. E. Clinical studies of viruses as antineoplastic agents with particular reference to Egypt 101 virus. *Cancer* 1952, 5, 1025-1034.
- Huebner, R. J.; Rowe, W. P.; Schatten, W. E.; Smith, R. R.; Thomas, L. B. Studies on the use of viruses in the treatment of carcinoma of the cervix. *Cancer* 1956, 9, 1211-1218.
- Russell, S. J. Replicating vectors for gene therapy of cancer: risks, limitations and prospects. *Eur. J. Cancer* 1994, 30A, 1165-1171.
- Yamanishi, K.; Takahashi, M.; Kurimura, T.; Ueda, S.; Minekawa, Y. Studies on live mumps virus vaccine. 3. Evaluation of newly developed live mumps virus vaccine. *Biken J.* 1970, 11, 157-161.
- Csatary, L. K.; Eckhardt, S.; Bukosza, I.; Czeglédi, F.; Fenyvesi, C.; Gergely, P.; Bodey, B.; Csatary, C. M. Attenuated veterinary virus vaccine for the treatment of cancer. *Cancer Detect. Prev.* 1993, 17, 619-627.
- Shimizu, Y.; Hasumi, K.; Okudaira, Y.; Yamanishi, K.; Takahashi, M. Immunotherapy of advanced gynecologic cancer patients utilizing mumps virus. *Cancer Detect. Prev.* 1988, 12, 487-495.
- Okuno, Y.; Asada, T.; Yamanishi, K.; Otsuka, T.; Takahashi, M.; Tanioka, T.; Aoyama, H.; Fukui, O.; Matsumoto, K.; Uemura, F.; Wada, A. Studies on the use of mumps virus for treatment of human cancer. *Biken J.* 1978, 21, 37-49.
- Heicappell, R.; Schirmacher, V.; von Hoegen, P.; Ahlert, T.; Appelhans, B. Prevention of metastatic spread by postoperative immunotherapy with virally modified autologous tumor cells. I. Parameters for optimal therapeutic effects. *Int. J. Cancer* 1986, 37, 569-577.
- European Medicines Agency (EMA). Report from the CPMP Gene Therapy Expert Group Meeting 26th-27th February 2004. EMA/CPMP/1879/04/Final, 2004.
- Ries, S. J.; Brandts, C. H. Oncolytic viruses for the treatment of cancer: current strategies and clinical trials. *Drug Discov. Today* 2004, 9, 759-768.