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RESEARCH PAPER

Assessment of Recovery Medium for Production of hCTLA4Ig after Cryopreservation in Transgenic Rice Cells

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Abstract A reproducible method for cryopreservation of transgenic rice cells (*Oryza sativa* L. cv. Dongjin) producing recombinant human cytotoxic T lymphocyte-associated antigen 4-immunoglobulin (hCTLA4Ig) has been established. Here, we assessed recovery media and investigated recombinant protein homogeneity after long-term preservation. For recovery of cryopreserved transgenic rice cells, AA medium was suitable in terms of both morphology and production of hCTLA4Ig. There were no differences in cell growth, sugar consumption, and hCTLA4Ig production between non-cryopreserved and cryopreserved cells for up to 1 month. hCTLA4Ig produced from cryopreserved cells was identical that of hCTLA4Ig from non-cryopreserved cells, as determined by analysis of its molecular weight and isoforms. For long-term preservation, cell viability was stably maintained at 61% for 26 months. In conclusion, these results demonstrate the possibility for reproducible cryocell-banking of transgenic rice cells without changes in the characteristics of cells and target proteins.

Keywords: cryopreservation, cryocell-banking, hCTLA4Ig, transgenic rice cells, two-dimensional electrophoresis

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1. Introduction

Recently, plant-made pharmaceuticals have become established as the next major commercial development due to the advantages of plant cells as hosts for the production of recombinant proteins, including safety without human pathogen, inexpensive media, and easy scale-up, making them more profitable compared with mammalian or human cells [1,2].

The rice expression system uses both the inducible RAmy3D promoter and signal peptide to produce high levels of target protein in culture medium under sugar starvation conditions [3]. Its high expression is attractive for the industrial production of biopharmaceuticals compared to other plant cells researched previously since materials are secreted into the cultured medium and the purification process is simple [4]. On the other hand, commercial production of recombinant proteins using plant cell cultures lacks a reliable methodology for cell-line preservation. Unlike mammalian cells and microbes, plant cells are only maintained by periodic subcultures, an issue that has often causes loss of productivity in high-producing cells over long culture periods. This type of maintenance also carries inherent problems such as time- or cost-consumption and contamination by mishandling [5]. Loss of the alkaloid-producing ability of *Cathranthus roseus* [6] and reduction of recombinant GM-CSF and IL-4 in *Nicotiana tabacum* after long-term subculture have been reported [7]. Therefore, long-term preservation of highly valuable cell lines is essential for transgenic plant cells, which are being considered for commercial use as industrial bioreactors [8]. In a previous study, we established a reproducible method for the cryogenic storage of transgenic rice cells, followed by successful recovery [9]. Growth characteristics of the recovered cells and the production level of recombinant

hCTLA4Ig after cryopreservation were confirmed. However, characterization of the recombinant hCTLA4Ig derived from recovered-cells has not been investigated.

In this study, we performed selection of recovered medium for long-term preservation of transgenic rice cells under cryogenic storage conditions. In addition, the growth properties of recovered cells were examined and characterization of secreted proteins analyzed using proteomic tools. Finally, we performed long-term preservation up to 1 year and determined post-thaw survival and genetic stability.

2. Materials and Methods

2.1. Rice cell cultures

Transgenic suspension cells of *Oryza sativa* L. cv. Dongjin producing 30 mg/L of hCTLA4Ig were established by Boryung Pharmaceutical Co. Ltd. (Ansan, Republic of Korea). Transgenic rice cell suspensions were cultured in modified AA medium composed of 2 mg/L of 2,4-dichlorophenoxyacetic acid, 0.2 mg/L of kinetin, 0.1 mg/L of gibberellic acid, 0.08 g/L of glycine, 0.88 mg/L of glutamine, 0.27 mg/L of aspartic acid, 0.23 mg/L of arginine and 3% (w/v) sucrose [10]. Hygromycin was added at a concentration of 50 mg/L as a selectable marker for maintenance of transformed rice cells. Cells were subcultured every 10 days and cultivated in a rotary shaking incubator at 110 rpm and 28°C under dark condition. The medium for hCTLA4Ig production was AA medium without sucrose.

2.2. Measurement of relative cell viability and cell growth

Viability of suspension-cultured rice cells was measured using a solution of triphenyl tetrazolium chloride (TTC) (pH 9.0). Fresh cells (0.1 g) was added to 1.6 mL of TTC solution to generate formazan and reacted at 20°C for 24 h. After the reaction solution was centrifuged at 16,074 × *g* for 10 min at 4°C, supernatant was removed. To extract formazan from viable cells, 1 mL of 95% ethanol was added at 60°C for 30 min. The supernatant was separated by centrifugation at 20,092 × *g* for 30 min at 4°C, and the extracted formazan was measured at 485 nm using a spectrophotometer (Agilent technologies, USA).

Dry cell weight (DCW) were measured for the estimation of cell growth. Suspension-cultured rice cells were filtered through Whatman No. 1 filter paper in a vacuum filtration apparatus and washed twice with distilled water. The washed cells were dried for 24 h at 60°C and then weighed.

2.3. Cryopreservation of transgenic rice cells

Suspension cells in exponential phase were pre-cultured for 5 days in AA medium containing 0.5 M sucrose to reduce water content in cells. Cryoprotectant was prepared from a mixture of 1 M DMSO, 1 M glycerol, and 1 M sucrose in N6 (Duchefa Biochemie, Netherland) medium with 2 mg/L of 2,4-dichlorophenoxyacetic acid. Pre-cultured cells (0.4 g) and 1.2 mL of cryoprotectants were added to CryoTube vials (Nunc, Denmark) to perform cryopreservation. Cryoprotected cells were placed in a Mr. Frosty™ Cryo 1°C freezing container (Nalgene, NY, USA) containing isopropyl alcohol to slowly freeze cells. Cells were immersed in liquid nitrogen after being pre-frozen for 2 h in a -70°C deep freezer. After 1 week of storage at -196°C, cells were rapidly thawed for 3 min in a 43°C water bath.

2.4. Post-thaw recovery and regrowth

Thawed cells were harvested, and cryoprotectant was immediately removed using Toyo No. 1 filter papers under low vacuum conditions. To recover cryopreserved rice cells, AA or N6 media (Duchefa biochemie, Netherlands) containing 2 mg/L of 2,4-dichlorophenoxyacetic acid, 0.5 g/L of proline, 0.5 g/L of glutamine, and 3% (w/v) sucrose were solidified with 0.8% (w/v) agar. Recovered cells after cryopreservation were also adapted to solidified AA medium and N6 medium containing 50 mg/L of hygromycin for 3 weeks. The regrown callus were induced separately into suspended cells in liquid AA medium and N6 medium, respectively.

2.5. Immunoassay

The hCTLA4Ig expression level in suspension-cultured medium was measured by sandwich enzyme-linked immunosorbent assay (ELISA). hCTLA4Ig was captured with goat anti-human IgG Fc (1:1,000, v/v; KPL, MD, USA) and blocked with phosphate-buffered saline containing 0.05% Tween 20 and 2% fetal bovine serum. The detection antibody goat anti-human IgG Fc (1:5,000, v/v; KPL, MD, USA) was used, after which horseradish-peroxidase (HRP) substrate 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS ; KPL, MD, USA) was added. ImmunoPure® Human IgG (PIERCE, MA, USA) from 11 to 0.08 ng/mL was used as a standard.

2.6. PCR Analysis

Genomic DNA was isolated from transgenic callus using a DNeasy plant mini-kit (Qiagen, German). PCR was performed using a reaction mixture containing Excel-*Taq* polymerase (CoreBioSystem, Korea) and two sets of primer pairs, hCTLA4Ig and hygromycin phosphotransferase: F1 (5'-TCCAACTTGACAGCCGGGGCAATGCACGTGGCCC AGCCTGC-3') is the N-terminus of CTLA-4 and R1 (5'-CTCTAGACTCATTTACCCGGAGACAGGGAG-3') is the C-terminus of the extracellular domain of IgG. Hyg-1 (5'-CTACATGGCGTGATTTCATA-3') is the N-terminus of hygromycin phosphotransferase and Hyg-2 (5'-CAC

TATCGGCGAGTACTTCT-3') is the C-terminus. Thermal cycling consisting of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C was repeated 30 times.

2.7. Reverse transcription PCR analysis

Total RNA was isolated from the cryo and non-cryo rice cells using an RNeasy Plant Mini Kit (Qiagen, Germany). 1 μg of total RNA was used to synthesize cDNA and PCR amplification using two step RT-PCR & GO kit (Q BIOgene, USA). The primer pairs used for CTLA4 mRNA expression analysis were as follows: hCTLA-4-F2 (5'-CTGCAGGCAATGCACGTGGCCCAG-3') and hCTLA-4-R2 (5'-GAACCGTGCCCAGATTCTGTCTAGA-3'). The rice β-actin was used as an internal control in gene expression studies.

2.8. Real-time quantitative PCR analysis

Genomic DNA samples for the quantitative RT-PCR analysis were extracted from the non-cryo and cryo rice cells using DNA extraction kit (DNeasy plant mini kit, Qiagen, Germany). The primer pairs used for qRT-PCR analysis were as follows: hCTLA-4-F3 (5'-TGCAAGGTGGAG CTCATGTA-3') and hCTLA-4-R3 (5'-AATCTGGGTTCC GTTGCCTA-3'). The rice UBQ5 was used as an internal control for calculation of transgene copy number [10]. TaqMan RT-PCR was performed in a thermal cycler (7300 Real time PCR system, Applied Biosystems, CA, USA). The amplification conditions consisted of one cycles of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C, 1 min at 60°C. Each sample was quantified in triplicate. The hCTLA4Ig copy numbers in genomic DNA was calculated using $2^{-\Delta\Delta CT}$ method [11].

2.9. Scanning electron microscopy

Callus tissues from AA medium and N6 medium were harvested and fixed with 2% glutaraldehyde (Sigma-Aldrich, MO, USA) in PBS (pH 7.0) for 4 h. The samples were rinsed three times with PBS buffer and dehydrated through an ethanol series (50 ~ 100%, v/v). Subsequently, these materials were dried in an air-flow for 30 min and coated with gold using a sputter coater to observe the extracellular space of cells. Scanning electron microscopy was performed using a Hitach S-700 scanning electron microscope (Hitach Koki Co., Japan) at an accelerating voltage of 15 kV.

2.10. Purification of hCTLA4Ig

hCTLA4Ig was purified using the rProtein A column (GE Healthcare Life Science, MA, USA). Culture medium was harvested 8 days after induction, followed by centrifugation for 30 min at 11,000 rpm for the removal of cell debris. Cultured medium was mixed with binding buffer (20 mM sodium phosphate buffer, pH 7.0) and purified using the ÄKTA FPLC system (GE Healthcare Life Science, MA, USA). The protein-bound rProtein A resin column was washed with 10 column volumes of binding buffer, and bound hCTLA4Ig was then eluted by 0.1 M glycine/HCl, pH 3.0. Fractions were collected and neutralized by the addition of 10 mM Tris/HCl buffer, pH 9.0. Fractions containing hCTLA4Ig were pooled and dialyzed using a Hiprep^{TM} 26/10 desalting column (GE Healthcare Life Science, MA, USA).

2.11. hCTLA4Ig Characterization

Molecular weight of hCTLA4Ig produced from cultured transgenic rice cells and recovered cells after cryopreservation were measured by mass spectrometry. The purified hCTLA4Ig was mixed with matrix solution consisting of saturated sinapic acid (3,5-demethoxy-4-hydroxycinnamic acid) in 50% acetonitrile containing 0.9% TFA and spotted onto MALDI plates. The spectra were collected on an Ettan MALDI-TOF/Pro system (GE Healthcare Life Science, MA, USA) operated in linear mode. MALDI-TOF was calibrated using BSA (Sigma-Aldrich, MO, USA) as a standard protein. The isoforms of expressed hCTLA4Ig were identified using two-dimensional electrophoresis. An immobilized pH gradient strip, pH 4 ~ 7, was rehydrated overnight with rehydration buffer consisting of 7 M urea, 2 M thiourea, 4% CHAPS, 100 mM DTE, and 40 mM Tris. Purified hCTLA4Ig was loaded into a sample cup, followed by isoelectric focusing (IEF) using IPGphor II (GE Healthcare Life Science, MA, USA). Focused strip gels were incubated for 40 min at room temperature with equilibration buffer consisting of 6 M urea, 20% glycerol, 2% SDS, 2.5% acrylamide solution, 200 mM Tris/HCl, pH 8.8, and 10 mM TBP. The second dimension was run on a 9 ~ 18% gradient gel and visualized using a modified Coomassie brilliant blue G-250 method.

3. Results

3.1. Assessment of recovery medium on cell morphology and hCTLA4Ig production after cryopreservation

Cryopreservation of transgenic rice cells was performed by following the optimum protocol we established in a previous study [9]. To verify changes in cell morphology during recovery period, post-thaw cells were recovered for 3 weeks in AA or N6 solidified medium. As shown in Figs. 1A and 1B, the recovered callus after cryopreservation for 1 week showed different phenotypes between AA and N6 solidified media. The recovered callus in N6 medium eventually aggregated and showed a browning phenomenon. Scanning electron microscopy was employed to investigate

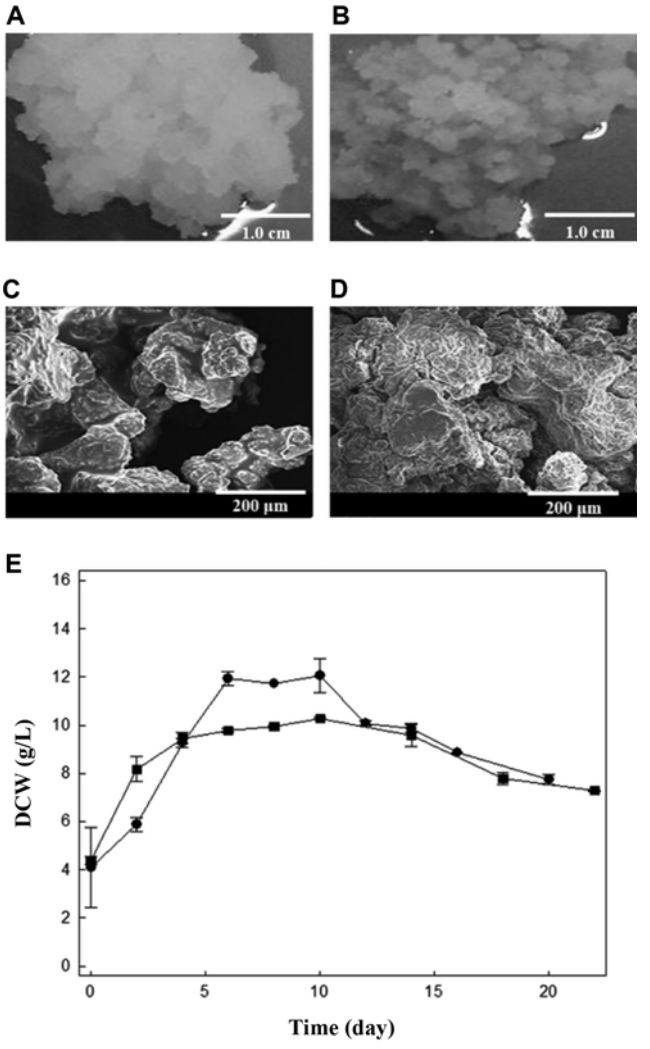

Fig. 1. Morphology of rice callus adapted in AA and N6 agar media after cryopreservation. Photography of rice callus cultured in (A) AA and (B) N6 media. Scanning electron microscopy of rice callus cultured in (C) AA and (D) N6 media. Time-course changes of (E) rice cell growth after cryopreservation in AA (■) and N6 (●) liquid media.

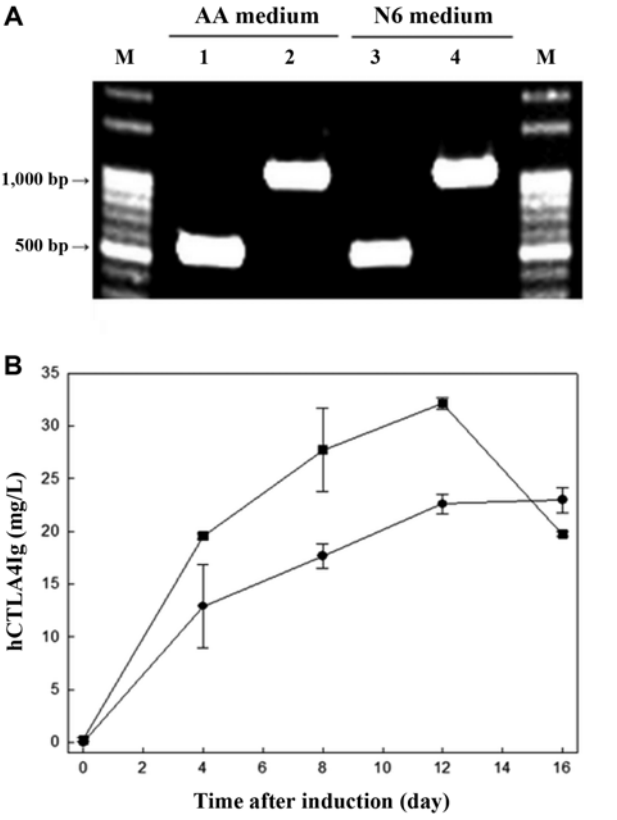

Fig. 2. Genomic DNA PCR of (A) hCTLA4Ig and Hyg^R from 1 week-cryopreserved callus; hygromycin phosphotransferase genes: M, 1 kb DNA ladder; lane 1, Hyg^R gene of regrown callus recovered in AA medium; lane 2, hCTLA4Ig gene of regrown callus recovered in AA medium; lane 3, Hyg^R gene of regrown callus recovered in N6 medium; lane 4, hCTLA4Ig gene of regrown callus recovered in N6 medium. Time-course changes of (B) extracellular hCTLA4Ig production after cryopreservation in AA (■) and N6 (●) liquid media.

morphologies of the regrown callus in both media (Figs. 1C and 1D). Under the same magnifying power, the regrown callus recovered from N6 medium showed greater aggregation than that recovered from AA medium.

The recovered callus in solidified medium for 3 weeks was induced into suspension cells in both AA and N6 media. Changes in the growth of recovered cells were measured in both medium containing 3% sucrose (Fig. 1E). Cells in AA medium grew without lag phase, and cell mass reached a maximum of 10 g/L after 8 days. However, in N6 medium, cells grew with lag phase for 2 days, after which maximum cell mass increased to 12 g/L after 8 days.

Next, we performed PCR analysis to determine genetic stability of the recovered callus in N6 and AA solidified media. As shown in Fig. 2A, hCTLA4Ig and hygromycin phosphotransferase genes from genomic DNA of the regrown callus recovered in both AA and N6 solidified media had lengths of 1,167 and 552 bp, respectively. Despite the recovered callus showing different phenotypes between AA and N6 solidified media, transgenes in the genomic DNA of rice cells were not affected by recovery medium.

To compare the biosynthetic capacity of cells in terms of recombinant proteins between AA and N6 media, expression of hCTLA4Ig was induced in sugar-free media since RAmy3D promoter is induced under sugar starvation conditions. Fig. 2B shows time-course changes in extracellular hCTLA4Ig production. The highest level of hCTLA4Ig production was obtained 12 days after induction in 32 mg/L of AA medium. On the other hand, 23 mg/L of hCTLA4Ig in N6 medium was produced 15 days after induction. These results suggest that use of AA medium as a recovery medium is suitable to maintain both morphology and

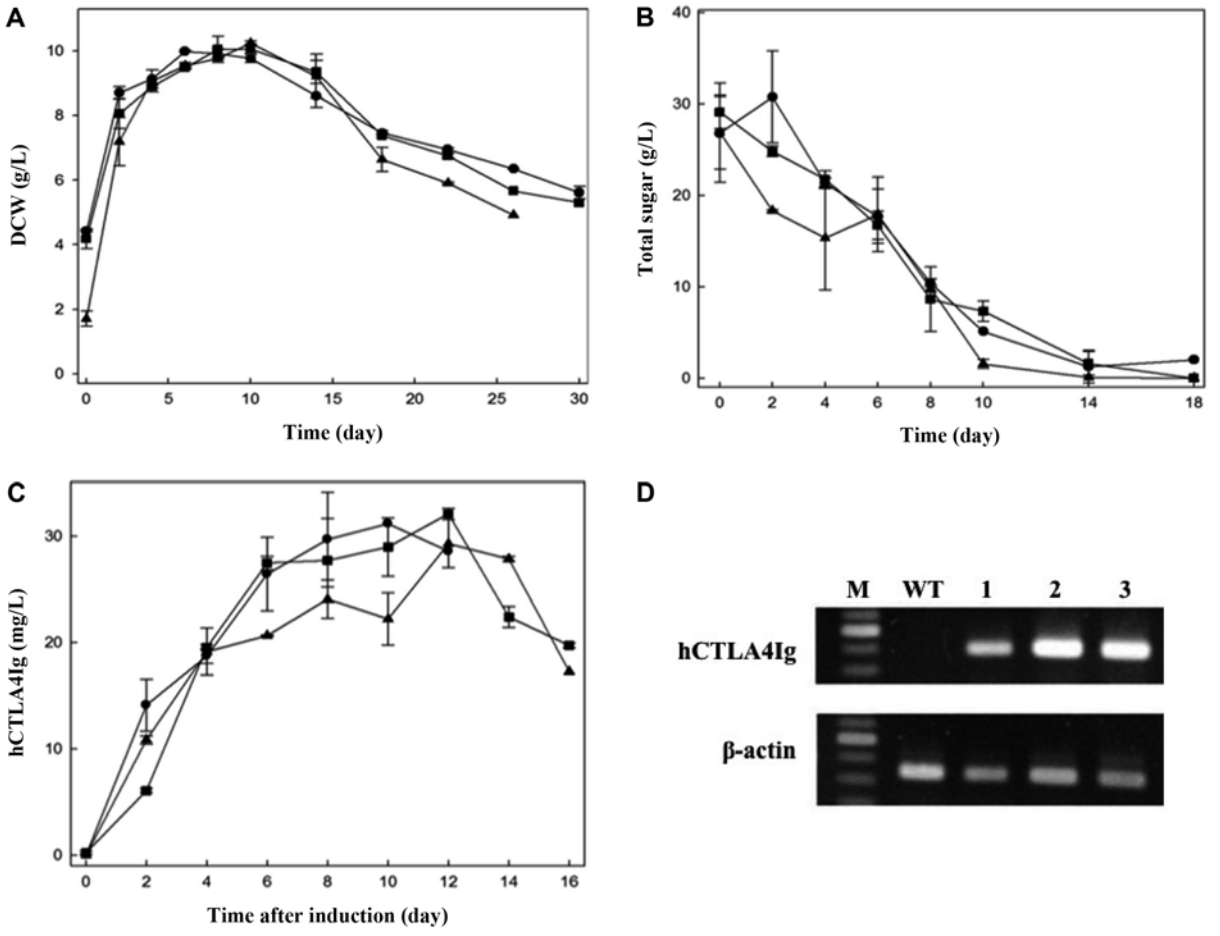

Fig. 3. Time-course changes of (A) rice cell growth, (B) sugar concentration, and (C) hCTLA4Ig productivity. Cells were cultured in AA medium containing 3% sucrose in a 100-mL flask. Sugar consumption in the cultured medium was analyzed using an HPLC system with a Zorbax carbohydrate column: ■, Non-cryopreserved cells; ▲, 1 week-cryopreserved cells; ●, 1 month-cryopreserved cells. This experiment was performed in duplicate. (D) mRNA expression of hCTLA4Ig and β-actin gene after post-thaw recovery: M, 1 kb DNA ladder; WT, wild type rice cells; lane 1, non-cryopreserved cells; lane 2, 1 week-cryopreserved cells; lane 3, 1 month-cryopreserved cells.

production of hCTLA4Ig of recovered cells.

3.2. Profiles of growth and hCTLA4Ig expression of recovered cells in AA medium

The growth and hCTLA4Ig production of cryopreserved cells recovered using AA solidified medium after 1 week and 1 month were verified and compared with those of non-cryopreserved cells. All recovered callus and non-cryopreserved cells grew similarly without lag phase, and the cell mass reached a maximum level of 10 g/L between 8 and 10 days (Fig. 3A). Sugar was similarly consumed during cell growth of cryopreserved and non-cryopreserved cells and exhausted after 14 days (Fig. 3B). The cells recovered after 1 week or 1 month cryopreservation showed no difference in terms of cell growth with non-cryopreserved cells.

The highest levels of hCTLA4Ig were obtained between 10 and 12 days after induction with 32, 29, and 31 mg/L of AA medium in non-cryopreserved, 1 week-cryopreserved, and 1 month-cryopreserved cells, respectively, after which hCTLA4Ig concentration decreased gradually (Fig. 3C). We also confirmed expression of the hCTLA4Ig gene using RT-PCR analysis. Expression levels of hCTLA4Ig mRNA against β-actin mRNA were maintained constitutively in both non-cryopreserved and cryopreserved cells (Fig. 3D). According to these results, the profiles of cell growth and the synthetic capacity of hCTLA4Ig in recovered cells after cryopreservation were similar to those in non-cryopreserved cells.

3.3. Isoform and molecular weight analyses of cryopreserved cell-derived recombinant hCTLA4Ig

To determine protein properties after cryopreservation, hCTLA4Igs were purified from non-cryopreserved and cryopreserved cells. Isoform and molecular weights of proteins were analyzed using one and two dimensional

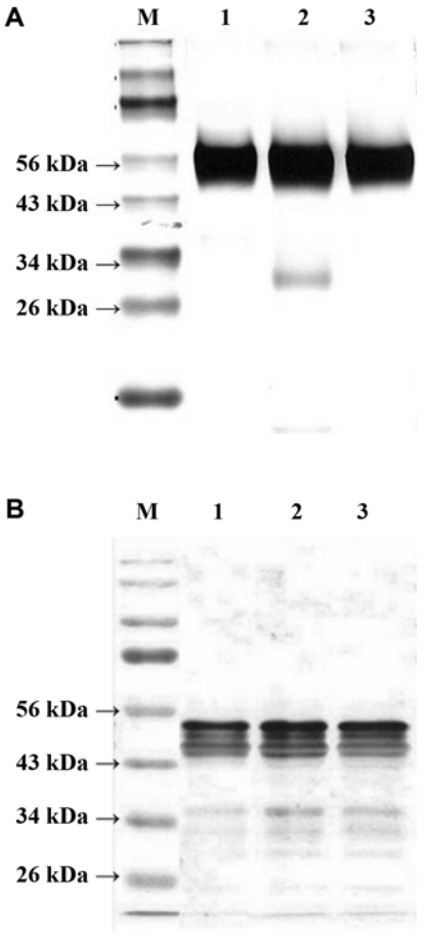

Fig. 4. (A) SDS-PAGE and (B) Western blot analysis of secreted hCTLA4Ig in sugar-free medium. Cultured medium was collected on day 10, and expressed hCTLA4Ig was detected with goat anti-human IgG antibody: M, PageRuler^{TM} Prestained Protein Ladder (Fermantas); lane 1, non-cryopreserved cells; lane 2, 1 week-cryopreserved cells; lane 3, 1 month-cryopreserved cells.

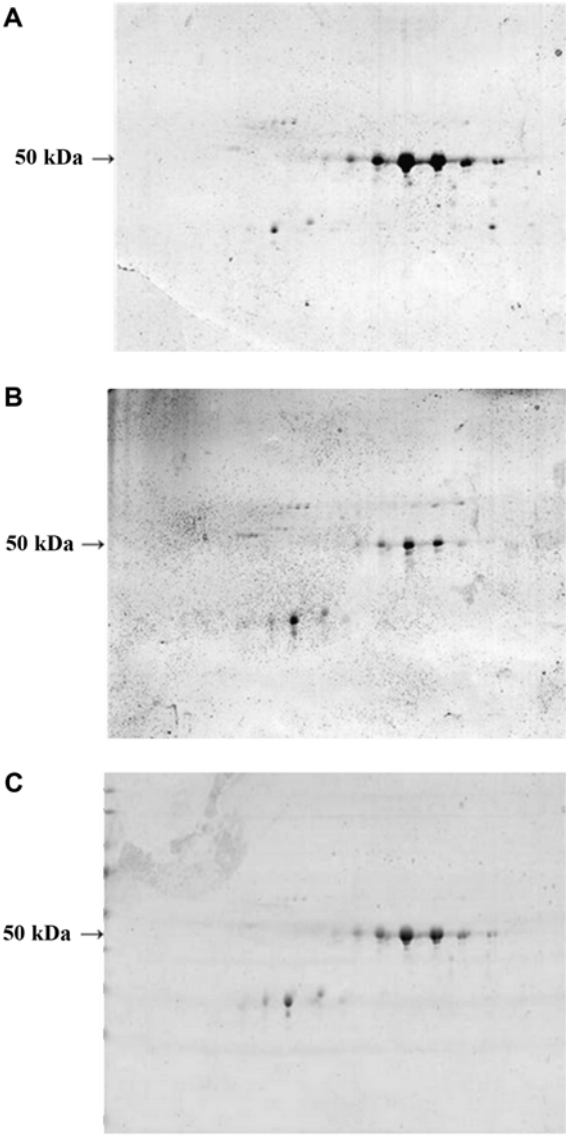

Fig. 5. 2D electrophoresis of purified hCTLA4Ig. The isoforms of hCTLA4Ig were separated by 2D electrophoresis. hCTLA4Ig (10 μg) was loaded on a rehydrated strip with the sample cup for isoelectric focusing (IEF), and the focused strip gel was then transferred for 2D-PAGE: (A), non-cryopreserved cells; (B), 1 week-cryopreserved cells; (C), 1 month-cryopreserved cells.

electrophoresis. hCTLA4Ig is a homodimer consisting of two identical subunits linked by disulfide bonds. The molecular weight of monomeric hCTLA4Ig in reduced gels was about 50 kDa as a single band in SDS-PAGE regardless of cryostorage (Fig. 4A). However, the result of Western blotting in reduced gels showed a main band and several minor bands in the range of 43 ~ 50 kDa (Fig. 4B). To compare its isoforms, hCTLA4Ig was separated by 2D electrophoresis (Fig. 5). In the pH range of 4 ~ 7, hCTLA4Ig isoforms showed two major spots and several minor spots. Major spots had a molecular weight of 50 kDa and were divided into several spots according to their isoelectric points. The isoform pattern of hCTLA4Ig derived from cryopreserved cells was the same as that of hCTLA4Ig derived from non-cryopreserved cells irrespective of storage duration. One and two dimensional electrophoresis provided an approximate comparison of the molecular weight of monomeric CTLA4Ig. For more accurate molecular weight analysis of hCTLA4Ig, purified recombinant proteins from non-cryopreserved and cryopreserved cells were measured by MALDI-TOF mass spectrometry (Fig. 6). The molecular weight of intact hCTLA4Ig was measured as 88 kDa by MALDI-TOF analysis, and the proteins confirmed identical size despite the cryostorage procedure.

3.4. Long-term stability of cryopreserved cells

Cryopreserved cells at -196°C were periodically recovered on AA solidified medium until 26 months, after which their

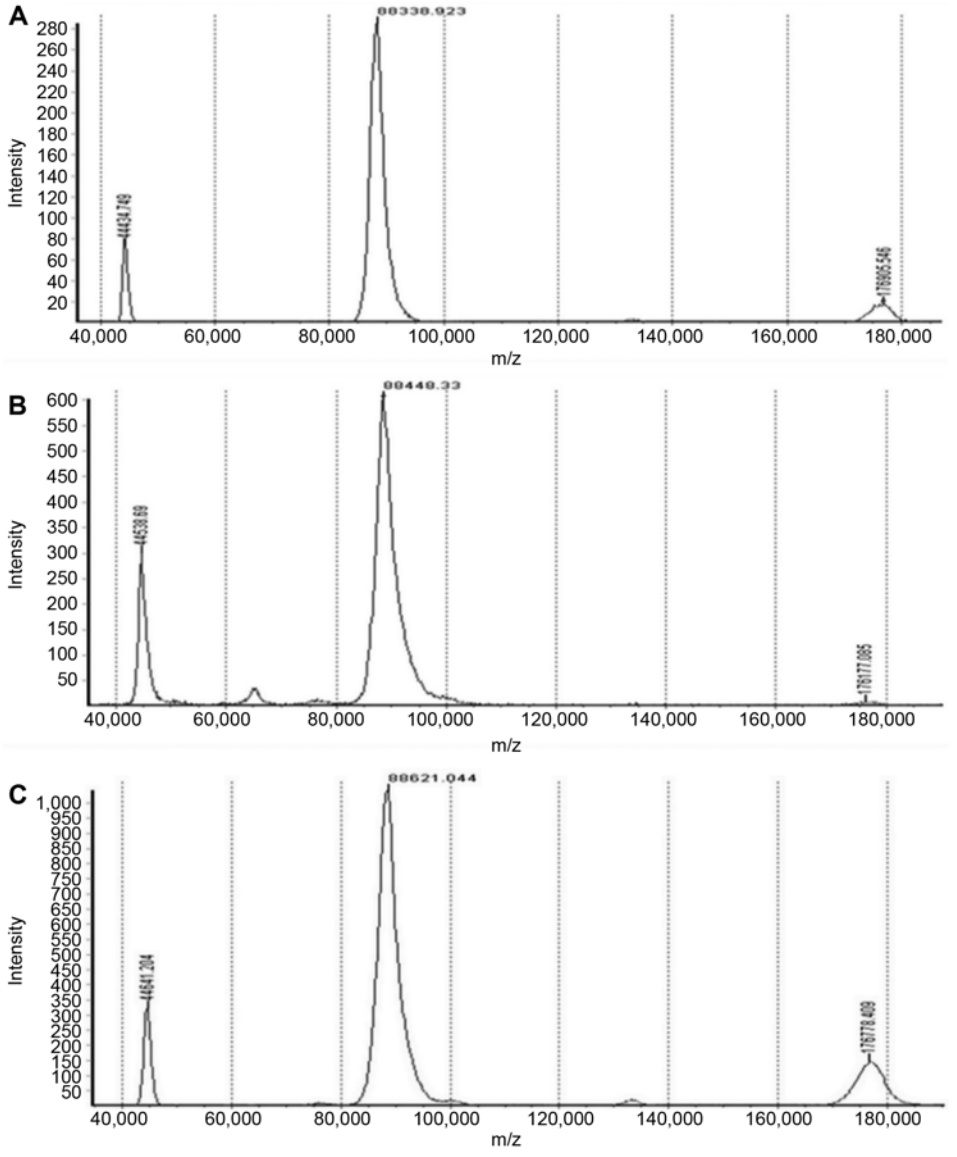

Fig. 6. MALDI-TOF mass spectra of purified hCTLA4Ig. The molecular mass of hCTLA4Ig was analyzed using MALDI-TOF in linear mode, and obtained mass spectra were calibrated using the system calibration. The first peak can be at half the m/z of the protein due to a double charge, and the second peak indicates the molecular weight of the protein. The third peak is an aggregate of the protein; (A), non-cryopreserved cells; (B), 1 week-cryopreserved cells; (C), 1 month-cryopreserved cells.

cell viability and transgene stability were measured. Initial post-thaw viability was 88% after 1 day of cryopreservation and decreased to 72% after 1 month. Cell viability did not decrease after 1 month and was maintained at 61% for 26 months (Fig. 7A). We isolated genomic DNA from the recovered callus and identified transgenes for hCTLA4Ig and hygromycin phosphotransferase using PCR analysis. As shown in Fig. 7B, the genes for hCTLA4Ig and hygromycin phosphotransferase from genomic DNAs of both non-cryopreserved and cryopreserved callus were clearly identified (1,167 and 552 bp, respectively). Additionally, transgene copy number and hCTLA4Ig productivity were estimated in non-cryopreserved and 1 year cryopreserved rice cells, respectively. As shown in Table 1, non-cryopreserve rice cell had about five copies of hCTLA4Ig gene that were also found to be stable after 1 year cryopreservation. hCTLA4Ig production was also maintained without reduced productivity in spite of 1 year cryopreservation (Fig. 7C).

4. Discussion

The key to successful plant cryostorage is reduction of damage during cryopreservation procedures [13]. In this

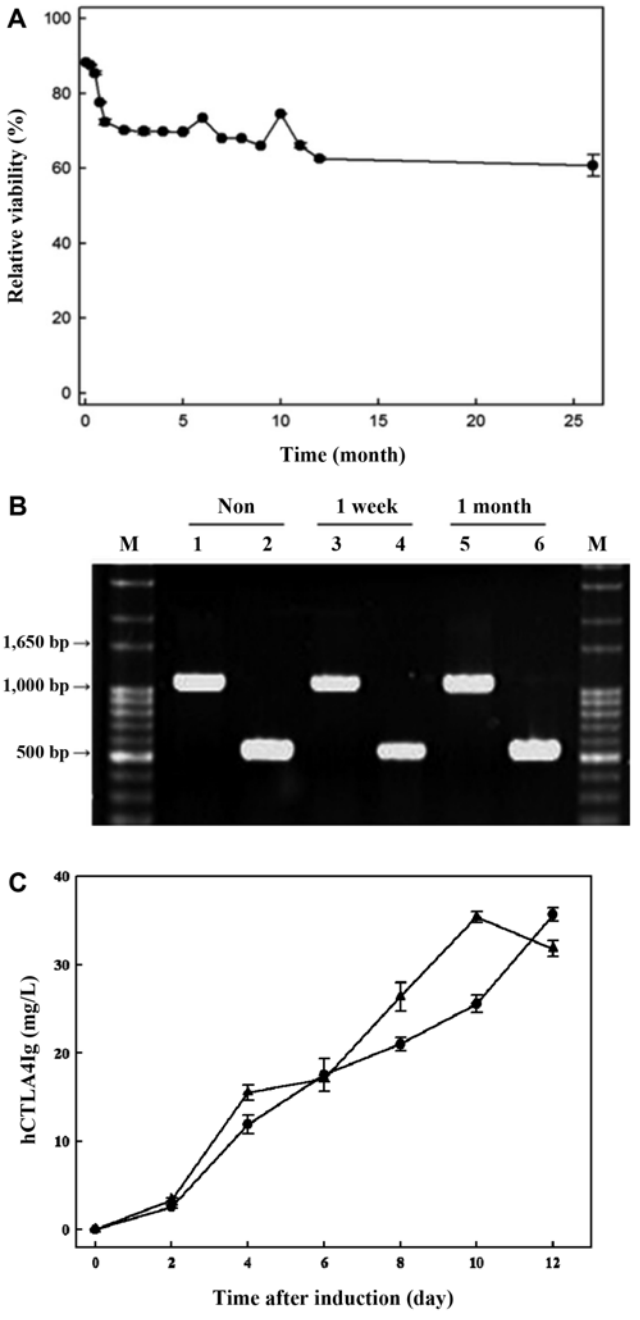

Fig. 7. Effect of storage period on cell viability, stability of transgenes and hCTLA4Ig productivity. (A) Cell viability was measured by TTC reduction assay, performed in triplicate. Pre-cultured cells were used as a control, and relative values were determined after cryopreservation. (B) Genomic DNA PCR of hCTLA4Ig and Hyg^R; hygromycin phosphotransferase genes: M, 1 kb DNA ladder; lane 1, hCTLA4Ig gene of non-cryopreserved cells; lane 2, Hyg^R gene of non-cryopreserved cells; lane 3, hCTLA4Ig gene of 1 week-cryopreserved cells; lane 4, Hyg^R gene of 1 week-cryopreserved cells; lane 5, hCTLA4Ig gene of 1 month-cryopreserved cells; lane 6, Hyg^R gene of 1 month-cryopreserved cells. (C) Cells were cultured in sugar-free AA medium in a 100 mL flask and measured hCTLA4Ig production using ELISA : ■, Non-cryopreserved cells; ▲, 1 year cryopreserved cells.

study, post-thaw cells were recovered in solidified medium for successful regrowth immediately after removal of the cryoprotectant. Plant cells after cryopreservation require a recovery period using solidified medium without shear stress due to weakening of cell walls during freezing and thawing procedures [14,15]. During this period, cell morphology is affected by recovery medium components since rice cells inherently aggregate in general culture medium. Rice cells are generally cultured in AA medium consisting of an amino acid nitrogen source or in N6 medium consisting of an inorganic nitrogen source [16]. Rice cells cultivated in these media tend to form aggregates, which may affect growth of rice cells or production of recombinant proteins [16]. Cell aggregation can also occur during the recovery period even though cells are fine before cryopreservation. Here, we confirmed whether or not cell aggregation affects growth and hCTLA4Ig production. Fig. 1A shows that the regrown callus after cryopreservation showed a significant difference in morphology between N6 medium and AA medium. Moreover, different phenotypes affected growth of cells and productivity of hCTLA4Ig in transgenic rice cells (Fig. 2). AA medium is composed of salts and amino acids such as aspartic acid, glutamine, arginine, and glycine and is known to dissociate cell wall polysaccharides to evoke a finely dispersed cell suspension [16]. In addition, arginine in AA media is metabolized in rice cells to form urea, which solubilizes polysaccharides during secretion [16]. Therefore, cells cultured using AA medium were apparently maintained as fine dispersed cells without removal of cell aggregates. Moreover, urea formed by cell metabolism in AA medium might induce secretion of recombinant proteins [17]. Consequently, use of AA medium as a recovery medium contributes not only to reduction of the recovery period after cryopreservation but also maintenance of healthy cells for high production of hCTLA4Ig.

Next, we measured cell growth and hCTLA4Ig accumulation in the recovered cells using AA medium after 1 week and 1 month of cryopreservation (Fig. 3). The growth of regrown cells was similar to that of non-cryopreserved cells, and the synthetic capacity of hCTLA4Ig was unaltered after cryopreservation. These results indicate that the growth kinetics and productivity of these cells were well preserved.

The hCTLA4Igs derived from cryopreserved and non-cryopreserved cells were separated by one and two dimensional electrophoresis (Figs. 4 and 5). The same sizes and isoform patterns of hCTLA4Igs suggests that the cellular process for production of recombinant protein was unaltered. Isoelectric point of mammalian cell derived hCTLA4Ig protein (abatacept) have been reported to be in the pH range of 4.5 ~ 5.7 which is very close to the

Table 1. Comparison of the transgene copy-number values estimated by RT-qPCR in non-cryopreserved and 1 year cryopreserved rice cells

Group	Amount of genomic DNA (ng)	Mean C_t		hCTLA4Ig copy number	Average hCTLA4Ig copy number
		UBQ5	hCTLA4		
Non-cryopreserved cells	100	16.31	13.71	6.09	5.38
	10	18.91	16.41	5.63	
	1	21.93	19.79	4.42	
1 year cryopreserved cells	100	16.38	13.66	6.59	5.66
	10	19.51	17.22	4.87	
	1	22.69	20.32	5.52	

theoretical pI (pI 5.67) based on its primary structure. However, we identified several isoforms of rice cell derived hCTLA4Ig in the pH range of 4 ~ 7 using 2D electrophoresis and several minor bands of 30 ~ 40 kDa at pH levels below 5 in SDS-PAGE. Recombinant hCTLA4Ig fusion protein-derived rice cells included a minor variant due to a complex glycosylation pattern such as recombinant β-secretase expressed in HEK-293 cells [18]. Moreover, these minor variants were not detected in the positive control-derived CHO cells (data not shown). The results show that the protein glycosylation pathway in the plant system was different from that in the CHO cell system [19 -21]. In particular, hCTLA4Ig has a total of three N-glycosylation sites, two of which are in the CTLA-4 sequence and one of which is from the Fc region of immunoglobulin [22]. N-glycans of proteins produced in transgenic plants include high mannose and complex type glycans lacking sialic acid [23-25]. Since hCTLA4Ig-derived rice cells contained N-glycosylation sites, it is possible that proteins of various sizes were present due to the N-glycan process.

Finally, we performed long-term preservation of cells and determined post-thaw survival rate in terms of cell growth (Fig. 7). The survival rate of cells after cryopreservation was maintained at above 60 percent during 26 months of storage. Additionally, the inserted genes and transgene copy numbers were stably maintained in genomic DNA of the recovered callus. In conclusion, these experimental evidences clearly suggest that cryocell-banking is the optimal procedure of stable preservation method for highly valuable cell lines in rice cell cultures.

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