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Preferential reduction of the α-2-6-sialylation from cell surface N-glycans of human diploid fibroblastic cells by *in vitro* aging

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Abstract Human diploid fibroblastic cell line, TIG-3, has a finite life span of about 80 population doubling levels (PDL), and is used for *in vitro* aging studies. Young cells (PDL 23) grew to higher cell densities at a higher growth rate than aged cells (PDL 77). When the electrophoretic mobility of cells was determined, the negative surface charge of the aged cells decreased significantly when compared to that of young cells. Lectin blot analysis of membrane glycoproteins showed that the α-2-6-sialylation but not the α-2-3-sialylation of N-glycans decreases markedly in the aged cells when compared to the young cells. In support of this observation, the cDNA microarray assay and reverse transcription-polymerase chain reaction (RT-PCR) analysis showed that the gene expression of the α-2,6-sialyltransferase I (ST6Gal I), which transfers sialic acid to galactose residues of N-glycans, decreases in the aged cells. These results indicate that the concordant decrease of the α-2,6-sialylation of N-glycans with the ST6Gal I gene expression is induced in TIG-3 cells by *in vitro* aging.

Keywords N-glycans · Sialylation · ST6Gal I · TIG-3 cells · *In vitro* aging

Abbreviations

CBB	Coomassie Brilliant Blue
Con A	concanavalin A
DT	doubling time
EtBr	ethidium bromide
β-1,4-GalT	β-1,4-galactosyltransferase
GlcNAcT II	UDP-GlcNAc
Manα	1,6-Man β-1,2-N-acetyl-glucosaminyltransferase

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G3PDH	glyceraldehyde 3-phosphate dehydrogenase
FCS	fetal calf serum
LCA	*Lens culinaris* agglutinin
L-PHA	leuko-agglutinating phytohemagglutinin
MAA	*Maackia amurensis* agglutinin
MES	2-(N-morpholino)ethansulfonic acid
PBS	phosphate-buffered saline
PDL	population doubling levels
PVL	*Psathyrella velutina* lectin
RCA-I	*Ricinus communis* agglutinin-I
RT-PCR	reverse transcription polymerase chain reaction
SNA	*Sambucus nigra* agglutinin
ST3Gal III	β-galactoside α-2,3-sialyltransferase III
ST3Gal IV	β-galactoside α-2,3-sialytransferase IV
ST6Gal I	β-galactoside α-2,6-sialyltransferase I

Introduction

The culture of normal human diploid fibroblastic cells has been used as a model for the aging of mitotic tissues and organs because the normal cells have the limited life span *in vitro*. In fact, the human diploid fibroblastic cell line, TIG-3, established in our institute cannot proliferate over the life span of about 80 population doubling levels (PDL), even under optimized culture conditions, unless they are immortalized. This replicative senescence of normal human diploid fibroblastic cells was first described by Hayflick and Moorhead [1], and was proposed to reflect processes that occur in aging of human organs and tissues [2,3]. However, the molecular mechanism that underlies cellular (*in vitro*) aging is still obscure. The shortening of telomere has been considered to be one of the potential mechanisms that explain cellular aging [4,5].

Our previous studies showed that N-glycosylation, particularly β-1,4-galactosylation, of human lung adenocarcinoma A549 cells changes markedly upon rapid cell senescence as induced by the treatment with transforming growth factor-β1 [6], which is independent of telomere-shortening [7], and augmented galactosylation and greater branching of N-glycans are induced in human stromal cells upon telomerase-expression [8]. These results suggest that changes in N-glycosylation occur during the course of cellular aging and most probably in *in vivo* aging, and could be important for aged cells, tissues and organs because N-glycans have been shown to play essential roles in the early development and growth of mammals [9–13]. In the present study, whether or not changes of N-glycosylation are induced in cell surface glycoproteins by cellular aging was investigated mainly by lectin blot analysis using membrane glycoprotein samples from young and aged TIG-3 cells.

Materials and methods

Cell culture

TIG-3 cells, established in Tokyo Metropolitan Institute of Gerontology, and human hepatoma cell line HepG2 cells were cultured in 60 mm plastic dishes containing 5 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), streptomycin (100 μg/ml) and penicillin (100 μU/ml) at 37°C under humidified 5% CO_2-95% air. Human Burkitt lymphoma cell line Raji cells were similarly cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), streptomycin (100 μg/ml) and penicillin (100 μU/ml). The cells were harvested by the treatment with 0.05% trypsin-0.53 mM EDTA at 37°C for 3 min. Cell number was counted with a hemocytometer, and cells were subcultured at a ratio of 1 : 4 once each week. To determine growth rate of cells, cells were placed at a density of 3.5×10^5 cells/60 mm dish in 5 ml DMEM containing 10% FCS. Medium was exchanged every 2 to 3 days, and cell number at each PDL was determined with a Coulter counter.

Chemicals

Horseradish peroxidase (HRP)-conjugated concanavalin A (Con A), *Ricinus communis* agglutinin-I (RCA-I) and Konica Immunostain HRP-1000 kit were obtained from Seikagaku Kogyo Co. (Tokyo, Japan). Biotinylated *Maackia amurensis* agglutinin (MAA), *Sambucus nigra* agglutinin (SNA), *Lens culinaris* agglutinin (LCA) and leuko-agglutinating phytohemagglutinin (L-PHA) were from EY Laboratories (San Mateo, CA). Biotinylated *Psathyrella velutina* lectin was a gift from Dr. N. Kochibe of Gunma University (Maebashi, Japan). HRP-conjugated streptavidin was from ZYMED Laboratory (South San Francisco, CA).

Lectin blot analysis of membrane glycoproteins

Membrane glycoprotein samples (15 μg protein) from TIG-3 cells at PDL 23 (PDL 23 cells) and TIG-3 cells at PDL 77 (PDL 77 cells) were subjected to 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a Mini Protean II Electrophoresis Cell (Bio-Rad Lab., Hurcules, CA), and proteins were transferred to polyvinylidene fluoride (PVDF) membranes using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Lab.). Lectin blot analysis was performed according to the method described previously [14]. In brief, blotted membranes were blocked with 1% bovine serum albumin for 2 h at room temperature and then incubated with 10 μg/ml HRP-conjugated Con A, RCA-I, L-PHA and

LCA or with biotinylated PVL, MAA and SNA followed by HRP-conjugated streptavidin. Biotin-conjugated lectins were further incubated with HRP-conjugated streptavidin. HRP-conjugated complexes were visualized with a Konica Immunostain HRP-1000 kit (Seikagaku Kogyo Co., Tokyo). In some experiments, blots were treated with 25 mM H_2SO_4 at 80°C for 60 min in order to remove sialic acid residues.

Determination of cell electrophoretic mobility

Cell electrophoretic mobility was determined according to the method described previously [15]. In brief, cells cultured for a week were harvested by the treatment with EDTA (37°C, 10 min) and suspended in 67 mM phosphate buffer containing 5.4% glucose (pH 7.3 and ionic strength 0.167) at approximately 1×10^6 cells/ml. The electrophoretic mobility of the cells was determined at 25 ± 0.5°C with a Cell Electrophoretic Microscope System (Model II V, Sugiura Laboratory Inc., Tokyo). The mobility of cells was calculated in μm/s/V/cm. Each cell mobility value was obtained by measuring the movements of at least 100 cells with reversal of polarity in each measurement. For accuracy of the system, the electrophoretic mobility of erythrocytes of normal male rats (Wistar, 4 weeks old) was also measured. Under these conditions, the mean value of rat erythrocyte mobility was -1.099 ± 0.028 μm/s/V/cm.

Analysis of gene expression using human cDNA microarrays

Poly(A)$^+$ RNA was isolated using a μMACS mRNA isolation kit (Miltenyi Biotec Inc., Bergisch Gladbach), and 1 μg of poly(A)$^+$ RNA was used for CyDye-labeled cDNA probe synthesis using a CyScribe First-Strand cDNA labeling kit (Amersham Biosciences Co., NJ). The cDNA probes from the PDL 23 and PDL 77 cells were labeled with Cy3 and Cy5, respectively. Two different dye-labeled probes were combined and subjected to cDNA microarrays containing approximately 1,000 human genes related to the syntheses and metabolisms of glycoconjugates and lipids supplied by Riken Institute (Saitama, Japan). After hybridization, the arrays were scanned by Affymetrix 428 Array Scanner (Affymetrix Inc., Santa Clara, CA), and the data obtained were analyzed by the ImaGene system (BioDiscovery Inc., El Segundol, CA). The same quantity of λpoly(A)$^+$RNA-A (Takara Bio Inc., Shiga, Japan) was added to both poly(A)$^+$ RNA samples as a control for correcting signals. A gene expression ratio (Cy5/Cy3) above 2.0 or below 0.5 was considered to be significant in the present study. Each experiment was conducted in duplicate.

RT-PCR analysis

Total RNA preparations were obtained from the PDL 23 and PDL 77 cells using a Sepasol (R)-RNA I reagent according to the manufacturer's instructions. The expression levels of the β-galactoside α-2,6-sialyltransferase I (ST6Gal I) gene, ribophorin I gene, N-acetylglucosaminyltransferase II (GlcNAc T II) gene, and β-galactoside α-2,3-sialyltransferase III (ST3Gal III) gene were analyzed by reverse transcription polymerase chain reaction (RT-PCR). RT-PCR analysis was conducted according to the method described previously [16] using the following 5′- and 3′-primers: 5′-TCTGGGCAAATGCTCTTCCTTGAG-3′ and 5′-ATTCTACCCAGTGTCGTCCCAGTCT-3′ for ST6Gal I; 5′-GCCCATCAGGCAGTGTTACCT-3′ and 5′-GCTAAAGGCAGAACCCAACCACC-3′ for ST3Gal III; 5′-GAATCCTGCTGCCTGCTGAGAAACA-3′ and 5′-AGTGGGCAGATTCAGGGTAGAAGAG-3′ for ST3Gal IV; 5′-CCTCCTTATTTTGGATGACTCTGTA-3′ and 5′-ATAACGGTGAAGAACAGGATGTAGA-3′ for ribophorin-I; 5′-CGTCCTCGTCATCTTTAGCCAT-3′ and 5′-ATGCCATAGAAACTGCGACTGG-3′ for GlcNAc T II; 5′-TCCACCACCCTGTTGCTGTA-3′ and 5′-ACCACAGTCCATGCCATCAC-3′ for G3PDH, and total RNA preparations as tamplates. RT-PCR thermocycling parameters were as follows: 60°C for 30 min; 94°C for 2 min; 32 cycles of 94°C for 1 min, 62°C for 90 sec; 62°C for 7 min for amplification of the ST6Gal I cDNA fragment, and 60°C for 30 min; 94°C for 2 min; 24, 24, 32, 32 and 24 cycles of 94°C for 1 min, 60°C for 90 sec; 62°C for 7 min for amplification of the ribophorin I, GlcNAc T II, ST3Gal III, ST3Gal IV, and dehydrogenase (G3PDH) cDNA fragments, respectively. The G3PDH gene was used as a control. The amplified products were analyzed on 2% agarose gel and stained with ethidium bromide (EtBr).

Analysis of hST6Gal I mRNA isoforms

Total RNA preparations were obtained from HepG2 cells, Raji cells, and PDLs 23 and 77 cells using an RNeasy Mini Kit (QIAGEN, Hilden, Germany). The first strand cDNA template was synthesized from 4 μg of total RNA using a Super Script III First-strand Synthesis System (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Four μl of the cDNAs were subjected to PCR analysis using specific primers as follows: pI, 5′-CTTTCTGTCTCTTATTTTTTGCCTTTGCAG-3′; pX, 5′-ACAACCAGGGAGGGCGTGGAAGCT-3′; pY, 5′-GCCCCGGCGTTAACAAAGGGAGCCG-3′; pII, 5′-TTCTTTTCCTTCCACACACAGATG-3′. PCR thermocycling parameters were as follows: 98°C for 30 sec; 35 cycles of 98°C for 10 sec, 67°C for 30 sec and 72°C for 30 sec; 72°C for 5 min.

Quantitative real-time RT-PCR analysis

Real-time RT-PCR analysis was performed with an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA) using qPCR™ QuickGoldStar Mastermix plus for SYBR Green I (Eurogentec, Seraing, Belgium). PCR primers were designed by the PRIMER EXPRESS software program (Applied Biosystems). Dissociation analysis was performed at the end of each PCR reaction. For 20 μl PCR assay, 1 μl cDNA template synthesized by a Super Script III First-strand Synthesis System (Invitrogen) was mixed with forward and reverse primers (500 nM each at the final concentration) and 10 μl qPCR™ QuickGoldStar Mastermix plus for SYBR Green I (Eurogentec). PCR thermocycling parameters were as follows: 95°C for 10 min; 40 cycles of 95°C for 15 sec and 60°C for 1 min. Gene-specific PCR was performed in duplicate on each cDNA sample. The gene-specific primers were as follows: hST6Gal I, 5′-CGCAGTCCTGAGGTTTAATGG-3′, 5′-CAGGCGAATGGTAGT- TTTTGTG-3′; hST3Gal III, 5′-TGGACAAACACTAGGCTCAGAGTATG-3′, 5′-CAGC-AGGCAGTTTAGAGTCCAGAT-3′; hST3Gal IV, 5′-ATGCAACAGCCACGGAAGAT-3′, 5′-GGAGGGCC-AGCGTGATG-3′; β-actin, 5′-AAGCCACCCCACTTCT-CTCTAAG-3′, 5′-AATGCTATCACCTCCCCTGTGT-3′. All sialyltransferase mRNA expression levels were shown as relative folds to that of the β-actin gene as described in user bulletin number 2 (Applied Biosystems).

Assay of sialyltransferase activities

Assay of ST6Gal I activity was performed as described previously [17]. TIG-3 cells were solubilized with 20 mM 2-(N-morpholino)ethansulfonic acid (MES) buffer (pH 6.5) containing 2% Triton X-100. Reaction mixture was incubated for 6 h at 37°C in a final volume of 50 μl of 0.1 M MES buffer (pH 6.5) containing 3 mM AMP, 250 μg asialo-fetuin, 1 mM CMP-[^{3}H]Neu5Ac (2,500 cpm/nmol) (PerkinElmer, Wellesley, MA), 10 mM $MnCl_2$, and 200 μg of cell homogenate proteins. The reaction was terminated by addition of 6 μl of 50% trichloroacetic acid, and precipitates were collected on Glass Microfiber Filter GF/C (Whatman, Middlesex, UK). The filters were washed with 5% trichloro-acetic acid twice and then with 95% ethanol once. Radioactivity was determined by liquid scintillation counter. Reaction mixture without acceptor was used as a control. Assays were performed in triplicate experiments. To determine amounts of α2-3-and α2-6-linked sialic acids, a portion of reaction mixture was treated initially with *Macrobdella decora* α-2,3-sialidase (Calbiochem, San Diego, CA) to remove α2-3-linked sialic acid, and then with *Arthrobacter ureafaciens* sialidase (Nacalai Tesque, Kyoto) to remove α2-6-linked sialic acid.

Results

Saturation cell density and growth rate of TIG-3 cells at each PDL

In order to examine whether or not saturation cell density changes by cellular aging, cell numbers of TIG-3 cells at the saturation density in each PDL were counted with a Coulter counter. The results showed that the cell density is relatively constant by PDL 50, but thereafter decreases rapidly by PDL 80, around which no more cells proliferated (Fig. 1). The saturation cell density at PDL 77 was approximately one-third of that of PDL 23 (Fig. 1). Therefore, at these PDLs, cell growth rates were examined by determining their doubling times (DT) in a logarithmic growth phase. The results showed that DT of PDL 23 cells is 21.1 ± 0.8 h, while that of PDL 77 cells is 35.1 ± 3.1 hr (Fig. 2). These results showed that the saturation cell density and growth rate of TIG-3 cells decrease significantly by cellular aging.

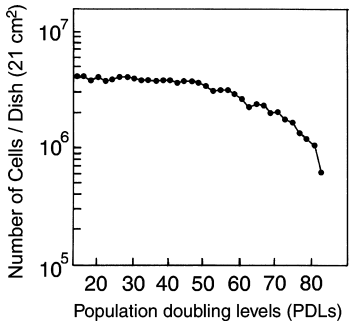

Fig. 1 Saturation cell densities of TIG-3 cells at each PDL. Cells were subcultured weekly at a ratio of 1:4, and cell numbers at saturation cell densities in each PDL were determined with a Coulter counter

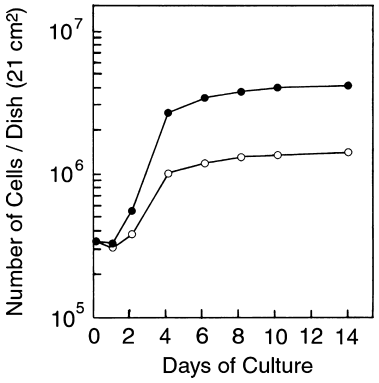

Fig. 2 Cell growth rates of PDL 23 and PDL 77 cells. Cell numbers of PDL 23 (closed circles) and PDL 77 (open circles) were counted periodically after subculture until reaching the saturation density

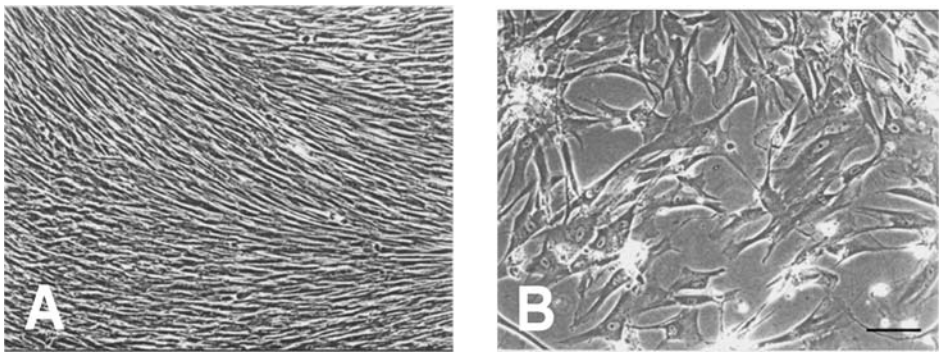

Fig. 3 Morphological appearances of PDL 23 (panel A) and PDL 77 (panel B) TIG-3 cells. Scale bar, 50 μm

Morphological appearances of PDL 23 and PDL 77 TIG-3 cells

As morphological changes are induced in the aged mammalian tissues and organs [18], the appearance of PDL 23 cells was compared with that of PDL 77 cells at the saturation cell density. PDL 23 cells showed a spindle-like appearance (Fig. 3A) while PDL 77 cells showed a flat shape with an increased volume (Fig. 3B). Moreover, cell-to-cell contact appeared tight in PDL 23 cells while that of PDL 77 cells was loose, indicating the differences in their cell surface properties including glycosylation of glycoproteins and glycolipids between PDL 23 and PDL 77 cells.

Lectin blot analysis of membrane glycoproteins from PDL 23 and PDL 77 cells

In order to examine whether or not cell surface glycosylation changes by cellular aging, membrane glycoprotein samples were prepared from PDL 23 and PDL 77 cells. They were subjected to SDS-PAGE, and proteins were transferred to PVDF membranes. When the blotted membrane was initially stained with Coomassie Brilliant Blue (CBB), protein components of both samples appeared similar except for a few protein bands with molecular weights of around 200 K, which were abundant in PDL 23 cells (lane A of Fig. 4 CBB). The glycosylation was analyzed by lectin blot analysis using a variety of lectins. When the blotted membrane was incubated with Con A, which interacts with high mannose-type and biantennary complex-type oligosaccharides [19], two bands with approximate molecular weights of 130 K (arrows in lane A of Fig. 4 Con A) disappeared in PDL 77 cell sample (lane B of Fig. 4 Con A). The blotted membrane was treated with mild acid to remove sialic acid residues, and then incubated with RCA-I, which interacts with oligosaccharides terminated with Galβ1-4GlcNAc/Glc group [20], and a slight but significant increase in the lectin binding was observed for most glycoprotein bands in PDL 77 cell sample (lane B of Fig. 4 RCA-I). The blotted membrane was incubated with L-PHA, which interacts with oligosaccharides with the Galβ1-4GlcNAcβ1-6(Galβ1-4GlcNAcβ1-2)Man branch [21], and most protein bands in PDL 77 cell sample reacted slightly stronger than those in PDL 23 cell sample (Fig. 4 L-PHA). Upon incubation of the blotted membrane with LCA, which interacts with oligosaccharides containing a fucose in the inner core [22], no significant difference was detected between two samples (Fig. 4 LCA). A similar result was obtained when the blotted membrane was incubated with PVL, which interacts with oligosaccharides terminated with β-*N*-acetylglucosamine residue [23] and also with α2-3-sialylated oligosaccharides [24] (Fig. 4 PVL). In the case of the sialylation of oligosaccharides, no change in the lectin binding was detected between two samples when the blotted membrane was incubated with MAA, which interacts with α2-3-sialylated oligosaccharides [25] (Fig. 4 MAA) but a remarkable decrease in the lectin binding was observed for most glycoprotein bands in PDL 77 cells when the blotted membrane was incubated with SNA, which interacts with

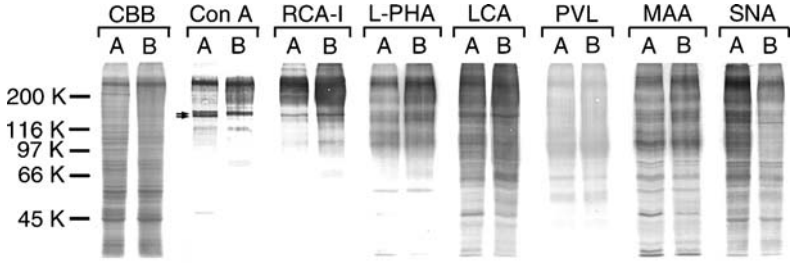

Fig. 4 Lectin blot analysis of membrane glycoprotein samples from TIG-3 cells. Lanes A and B indicate those from PDL 23 and PDL 77 cells, respectively. The blotted membrane was incubated with CBB, Con A, RCA-I, L-PHA, LCA, PVL, MAA or SNA to detect protein bands or reacting oligosaccharides

α2-6-sialylated oligosaccharides [26] (Fig. 4 SNA). The MAA- and/or SNA-positive bands disappeared upon treatment of the blots with *Arthrobacter ureafaciens* sialidase (data not shown). Furthermore, most protein bands positive to lectins used in the present study disappeared upon treatment of the blots with N-glycanase (data not shown), indicating that the glycosylation patterns analyzed with lectins in the present study are those of N-glycans. These results indicate that changes of N-glycosylation, particularly a decrease of the α-2,6-sialylation, occur in TIG-3 cells by cellular aging.

Cell surface negative charges of PDL 23 and PDL 77 cells

As the α2-6-sialylation of N-glycans decreased markedly from the membrane glycoprotein sample of PDL 77 cells (lane B of Fig. 4 SNA), whether or not the cell surface negative charges of TIG-3 cells decrease by cellular aging was examined between PDL 23 and PDL 77 cells by cell electrophoresis. The results showed that the mean electrophoretic mobility is -1.581 ± 0.097 μm/sec/V/cm for PDL 23 cells and -1.326 ± 0.087 μm/sec/V/cm for PDL 77 cells, respectively (Fig. 5), indicating that the net negative charge of the cell surface decreases by cellular aging.

Gene expression levels of glycosyltransferases

Since several types of N-glycosylation changed by cellular aging as revealed by lectin blot analysis, the gene expression levels of enzymes that are involved in N-glycan biosynthesis were determined between PDL 23 and PDL 77 cells using human cDNA microarrays which contain about 1,000 genes related to proteins for the glycoconjugate biosynthesis, kindly provided by RIKEN Institute. Significant decreases were observed for the genes that encode ST6Gal I, ribophorin-I and GlcNAcT II in PDL 77 cells when compared to PDL 23 cells (Table 1). In the case of the ST6GalT I, the expression level was down-regulated to one-fourth in PDL 77 cells while those of the ST3Gal III and ST3Gal IV were unchanged between PDL 23 and PDL 77 cells. Similarly, in the case of the ribophorin-I, one of the components that form oligosaccharyl transferase [27], and GlcNAcT II, their gene expression levels in PDL 77 cells decreased to one halve to one-third of those of PDL 23 cells.

Table 1 Gene expression of glycosyltransferases and related protein in TIG-3 cells

Genes	Ratio (PDL 77/PDL 23)
ST6Gal I	0.22 ± 0.05
ST3Gal III	–
ST3Gal IV	–
Ribophorin-I	0.47
GlcNAc T II	0.36 ± 0.10
β-1,4-Gal T VII	–

Changes in gene expression of PDL 77 greater than 2-fold above or below that of PDL 23 were considered significantly. The results were shown in the mean value of the duplicate experiments.
–: Not changed significantly.

To confirm the gene expression levels as obtained using the cDNA microarrays, RT-PCR analysis was conducted using specific primers. The results showed that the gene expression levels of the ST6GalT I (panel a), ribophorin-I (panel d) and GlcNAcT II (panel e) decrease and those of the ST3GalT III (panel b) and ST3GalT IV (panel c) increase in PDL 77 cells when compared to those of PDL 23 cells (Fig. 6). Although the results from cDNA microarrays and lectin blot analysis showed no significant changes in the gene expression levels of enzymes that are involved in the

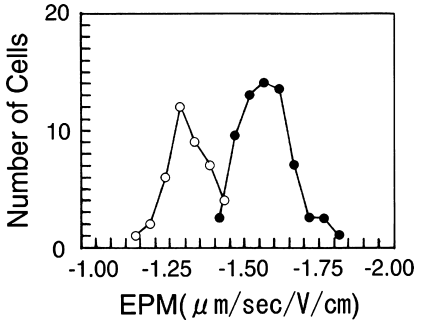

Fig. 5 Electrophoretic mobility of TIG-3 cells. The electrophoretic mobility of PDL 23 (closed circles) and PDL 77 (open circles) on day 7 after subculture were determined by cell electrophoresis, and are expressed as electrophoretic mobility (EPM: μm/sec/V/cm), a term often used in this type of study [15].

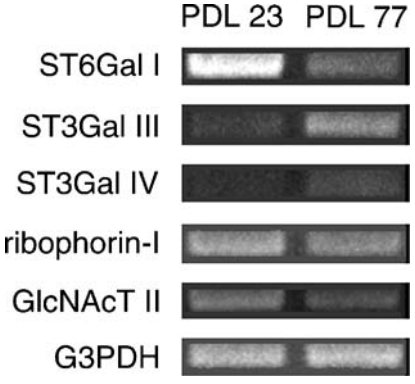

Fig. 6 Comparison of the expression levels of the glycosyltransferase genes between PDL 23 and PDL 77 cells. RT-PCR was carried out using total RNA from PDL 23 (lane A) and PDL 77 (lane B) cell preparations and oligonucleotide primers specific to ST6Gal, ST3Gal III, ST3Gal IV, ribophorin-I, GlcNAcT II and G3PDH genes. The PCR products were visualized on 2% agarose gel stained with EtBr. The analysis was conducted three times, and identical results were obtained.

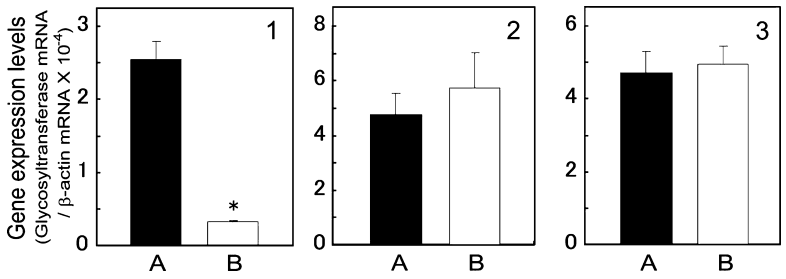

Fig. 7 Relative gene expression levels of ST6Gal I (panel 1), ST3Gal III (panel 2) and ST3Gal IV (panel 3) to that of β-actin gene between PDLs 23 and 77 cells. Quantitative real time RT-PCR analysis was performed using total RNA preparations from PDL 23 cells (A) and PDL 77 cells (B). The results show means ±S.D. of four separate experiments. * indicates $p < 0.00001$.

α2-3-sialylation of glycoproteins and actual product α2-3-sialic acid residues appeared unchanged by cellular aging, but their gene expression levels were shown to be slightly increased by PCR-analysis. To confirm this further, quantitative real time RT-PCR was performed using total RNA preparations from PDL 23 and 77 cells. The results showed that the gene expression level of the ST6Gal I decreases to one-tenth in PDL 77 cells when compared to that of PDL 23 cells without changing the levels of the ST3Gal III and ST3Gal IV (Fig. 7), which is consistent to those shown in Fig. 6.

Sialyltransferase activities in TIG-3 cells

As the α2-6-sialylation of N-glycans and the gene expression level of ST6Gal I decreased by cellular aging, whether or not α2-6-sialyltransferase activity also decreases was examined using asialo-fetuin as a sugar acceptor in PDLs 23 and 77 cells. The results showed that α2-6-sialyltransferase activity in PDL 77 cells decreases by 40% when compared with PDL 23 cells while no significant change is observed for α2-3-sialyltransferase activity between PDLs 23 and 77 cells (Fig. 8), indicating that the enzymatic activity also decreases by cellular aging.

Isoforms of the ST6Gal I transcript in TIG-3 cells

In humans, there are isoforms of the ST6Gal I mRNA produced by alternative splicing of the gene [28]. At least three different promoters have been described to control the tissue-specific expression of the transcripts. The form 1 is expressed predominantly in liver, form 2 in B cell lines, and form 3 in many other tissues [29–32]. In order to investigate which isoform is expressed in TIG-3 cells, RT-PCR analysis was performed in PDLs 23 and 77 cells using three different sets of primers (pI and pII, pX and pII, and pY and pII) specific to the individual isoforms (Fig. 9 panel A). When RT-PCR analysis was conducted in HepG2 cells and Raji cells as controls, forms 1 and 3 were expressed in HepG2 cells (lane 1 of Fig. 9B), and forms 2 and 3 were expressed in Raji cells (lane 2 of Fig. 9B), respectively. In the case of TIG-3 cells, only form 3 was expressed both in PDLs 23 and 77 cells (lanes 3 and 4, respectively, of Fig. 9B), and that the expression level of form 3 decreases in PDL 77 cells when compared with PDL 23 cells (lanes 3 and 4 of Fig. 9B).

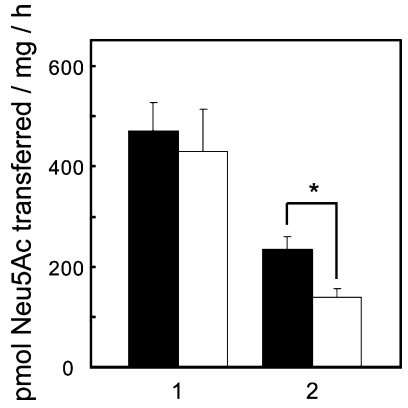

Fig. 8 Sialyltransferase activities in PDL 23 cells (■) and PDL 77 cells (□). Sialyltransferase assays were performed for 6h in the presence of CMP-[^{3}H]neuraminic acid and asialo-fetuin, and then [^{3}H]-labeled glycoprotein was initially digested with *Macrobdella decora* α2-3-sialidase to remove α-2,3-linked sialic acid and then with *Arthrobacter ureafaciens* sialidase to remove α2-6-linked sialic acid for determining the amounts of α2-3-linked and α2-6-linked sialic acids (Neu5Ac). 1, α2-3-linked Neu5Ac; 2, α2-6-linked Neu5Ac. The results show means ±S.D. of three separate experiments. * indicates $p < 0.01$

Discussion

The aged (senescent) cells have a variety of characteristic features such as enlarged and flattened shape, resistance to mitogen-activated cell proliferation, expression of the senescence-associated β-galactosidase, and loss of cell contact [1–3, 33]. Age-associated changes of N-glycans, gangliosides and proteoglycans have been described in human brain, serum IgG, erythrocytes and fibroblastic cells [34–42]. However, no detailed study on N-glycans during cellular aging was conducted.

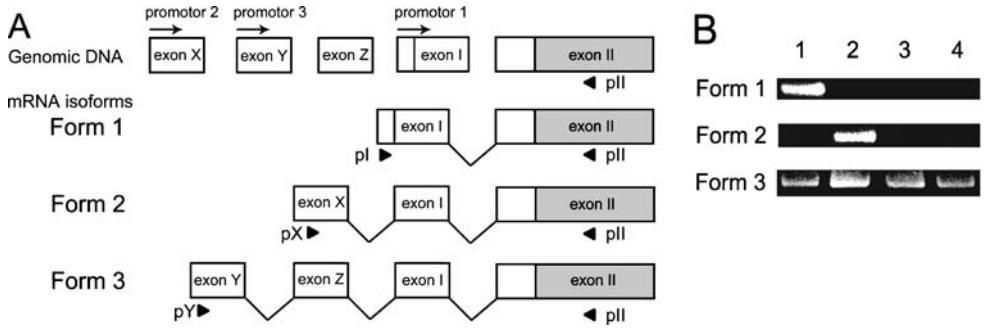

Fig. 9 RT-PCR analysis of hST6Gal I mRNA isoforms in TIG-3 cells. Panel A shows the genomic organization of the 5′-untranslated region of the *hST6Gal I* and resultant hST6Gal I mRNA transcripts. Open and gray boxes indicate the 5′-untranslated regions and coding region, respectively. The arrowheads denote primers used for RT-PCR analysis. Panel B shows the results obtained by RT-PCR analysis using total RNA preparations from HepG2 cells (lane 1), Raji cells (lane2), PDL 23 cells (lane 3) and PDL 77 cells (lane 4). HepG2 cells (lane 1) and Raji cells (lane 2) were used as controls for form 1 and form 2, respectively

Our previous study showed that N-glycosylation of human stromal cells changed by telomerase expression [8] and galactosylation of N-glycans changed in human lung adenocarcinoma A549 by rapid cell senescence as induced by the treatment with transforming growth factor-β1 [6]. The present study showed that N-glycosylation, particularly α2-6-sialylation of N-glycans of TIG-3 cells dramatically decreases upon cellular aging as shown at the gene expression and enzymatic levels. The reduced sialylation in senescent cells was initially reported in human and rat erythrocytes [43,44], but these studies did not show what type of sialic acid residues changed. The linkages of sialic acid can be classified into three groups: α2-3-, α2-6- and α2-8-linkages. The α2-8-linked sialic acid often occurs as polysialic acids and oligosialic acids, and their expression is rather limited to certain glycoproteins and regulated spatially and temporally [45–47]. In contrast, α2-3-linked and α2-6-linked sialic acids are expressed abundantly on cell surface glycoproteins and glycolipids. In the present study, it was shown that α2-6-linked sialic acids but not α2-3-linked sialic acids of N-glycans decrease markedly by cellular aging. The α2-6-linked sialic acids of N-glycans (Neu5Acα2-6Galβ1-) are synthesized by ST6Gal I and ST6Gal II. The ST6Gal I gene is expressed in most tissues, while the ST6Gal II gene is expressed only in small intestine, colon and fetal brain [48]. In humans, there are at least three isoforms of the ST6Gal I transcript; form 1 is expressed in liver, form 2 in B cell lines, and form 3 in many other tissues [28–32]. RT-PCR analysis showed that only form 3 is expressed in TIG-3 cells and that the expression level decreases in PDL 77 cells when compared with PDL 23 cells. Since our histochemical study showed that the α2-6-linked sialic acids also decrease in mouse hepatocyte cell surface glycoproteins by *in vivo* aging (Funahashi H., and Furukawa K, unpublished data), the decrease of the α2-6-linked sialic acids may occur in many aged tissues and organs. Senescent cells, apoptotic cells and injured cells are removed by macrophages *in vivo* [49]. Macrophages recognize these target cells with C-type lectins such as macrophage Gal/GalNAc-specific lectin and mannose receptor [50,51]. Since the loss of terminal sialic acid residues results in the expression of galactose residues, it is rational that the exposed galactose residues are involved in the phagocytosis of senescent cells. Moreover, the loss of α2-6-sialic acid residues from glycoproteins may also affect cell proliferation and cellular interactions including cell-to-cell and cell-to-matrix interactions, the mechanism of which remains to be clarified [52–55]. A further study is necessary for elucidating biological significance of the α2-6-sialylation that decreases by cellular aging by exploring receptors for α2-6-linked sialic acids if any expressed at cell surface of TIG-3 cells and by determining what kind of a signal is induced upon their binding. Alternatively, the overexpression or silencing of the ST6Gal I gene in the cells may afford some clues to cellular aging. As sialic acid residues of glycoproteins and glycolipids and sulfate and carboxyl groups of proteoglycan are main contributors to negative charges at cell surface, a decrease in the α2-6-siaylation of N-glycans is not all but a significant part of the decrease of the negative charges by cellular aging, and may affect the events occurred at cell surface.

Slight but significant decreases of the expression of the ribophorin-I and GlcNAcT II genes and less binding of Con A to glycoproteins in PDL 77 cells as compared to those in PDL 23 cells could be ascribed to reduced N-glycosylation of proteins in aged cells. Therefore, it will be of interest to determine the number of N-glycans attached to a particular glycoprotein produced in aged cells and compare with that produced in young cells. No significant change was observed between PDL 23 and PDL 77 cells in the expression levels of the β1,4-galactosyltransferase (β1,4-GalT) genes. In the case of the rapid cell senescence, the expression levels of the β1,4-GalT III, IV and VI changed significantly with altered galactosylation of membrane glycoproteins [6]. Concerning to the β1,4-GalT VII, a point mutation of this

gene was shown to be responsible for progeroid variant of Ehlers-Danlos syndrome [56–58], but no change of this gene expression was associated with this cellular aging. A further study is necessary to elucidate biological relevance of the PDL-dependent variation of N-glycosylation in *in vivo* aging.

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