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Senescence-induced alteration in cell surface carbohydrates correlated using proton NMR spectroscopy and a lectin-based affinity-binding assay

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Changes in the cell surface oligosaccharides in human fetal lung fibroblasts (IMR-90) are studied as the cells progress to senescence using nuclear magnetic resonance spectroscopy (NMR) and a biochemical assay. A lectin-based affinity-binding technique is used which measures the organization of carbohydrates on the cell surface. Proton NMR studies of the water in samples of frozen cell suspensions of young and old cells provide information on the local dynamics of the cell surface by monitoring the motion of bound water. Changes in the lectin binding density and affinity class distribution correlate with a decrease in the water proton linewidth in frozen cells. These observations reflect alterations in the conformation or structure of the cell surface oligosaccharides and local constituent water.

Introduction

The cell surface oligosaccharides have been shown to be important in developmental, maturation, and transformation processes. The surface oligosaccharides direct the developmental staging and adhesion-dependent differentiation in *Dictyostelium* cells [1,2]. The oligosaccharides have also been shown to be important in epithelial migration and in the enunciation of functional cytotoxicity [3–5]. Tumor-specific surface markers have been shown to be altered oligosaccharide residues [6,7].

In three previous studies human fetal lung fibroblasts (IMR-90) were shown to undergo a complex series of changes in cell surface oligosaccharides as the cells progress to senescence [8–10]. The studies used lectin-based binding assays to observe the interactive properties of the cell surface oligosaccharides. These properties are defined by specificity, affinity of interaction, and the binding capacity. Three different lectins were used in the studies: *Ricinus communis* agglutinin, (RCA-120) which is specific for β-D-galactose; concanavalin A (ConA) which is specific for α-D-glucosyl and mannosyl sugars; and the wheat germ agglutinin (WGA), which is specific for *N*-acetyl-D-glucosamine. Different changes were observed for each of the lectins. These were changes or losses of affinity class distribution, changes in binding capacity, and changes in lectin-membrane complex mobility. The observations from the lectin assays reflect changes in the structure or conformation of the cell surface oligosaccharides.

In this study a nuclear magnetic resonance (NMR) technique is used to define structural/conformational changes that take place in the cell surface oligosaccharides as IMR-90 cells progress toward senescence from the properties of interacting water molecules. The proton NMR spectra of cells are very complicated and little meaningful information can be obtained directly from residues on the cell surface. This paper describes an indirect method to study the structure and conformation of cell surface oligosaccharides from measurements of the relaxation properties of the water protons associated with the oligosaccharides. The spin-lattice (T_1) and spin-spin (T_2) relaxation times of water are influenced greatly by interaction with macromolecules in solution [11]. As water binds to the macromolecule the effective correlation time is changed, which reduces the spin-spin relaxation time. In a solution of water and a macromolecule, the observed relaxation time is a weighted average of the relaxation times of the bulk water and the bound water [12].

Abbreviations: ConA, concanavalin A (biotinylated lectin); PBS, phosphate-buffered saline; BSA, bovine serum albumin.

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The measurement of the relaxation time of the bound water is complicated by the contributions from the bulk water. This can be overcome by freezing the sample. When a solution of water and a macromolecule is frozen, the signal from bulk water is not observed. However, a peak is detected from the associated water which does not freeze because the macromolecule disrupts the crystal lattice of the water [13]. The linewidth (T_2^*) of this peak is sensitive to the conformation of the macromolecule. Kuntz has shown that for frozen solutions of polypeptides, those in the helical conformation exhibit much broader lines than when studied in the random coil conformation [14]. In another study Zipp et al. [15] showed that the relaxation rate of the bound water in frozen samples of normal and sickle erythrocytes is sensitive to the structural differences between the two cells.

In the present study, cell suspensions of IMR-90 cells were frozen at different points in their lifecycle and the linewidth of the water protons was measured. Changes in the linewidths are correlated with alterations in the display of oligosaccharides on the cell surface [8–10]. The origin of the change in the water signal is confirmed through treatment of cells with *t*-butanol and swainsonine. The *t*-butanol treatment has been shown to remove small amounts (approx. 1 pg per cell) of cell surface glycoproteins without damaging the cells [16]. The treatment of the cells with swainsonine inhibits the Golgi enzyme α-mannosidase II and results in the formation of altered hybrid-type oligosaccharides on the cell surface [17]. Both the *t*-butanol and swainsonine treatments of the IMR-90 cells change the carbohydrate structure on the cell surface compared to the untreated cells, and the alteration is observed as a perturbation in the linewidth of the unfrozen water.

Materials and Methods

Cell culture and sample preparation

The IMR-90 cells were grown and maintained as described previously [8–10]. Prior to freezing cell cultures at 80% surface confluency were washed three times with Ca/Mg free phosphate-buffered saline (PBS), containing 2.7 mM KCl, 1.5 mM KH_2PO_4, 80 mM Na_2HPO_4, and 0.137 M NaCl (pH 7.2). The cultures were then incubated with 0.25% trypsin (Flow Lab) at 310 K for 2–3 minutes. After transfering the cell suspensions to centrifuge tubes the cells were washed twice with complete medium [8], counted and resuspended in complete medium for 60–90 min. Aliquots of $(2–3) \cdot 10^7$ cells were resuspended in complete medium supplemented with 10% dimethyl sulfoxide (DMSO) (Sigma) then placed in 5-mm NMR sample tubes, and centrifuged at $100 \times g$ for 5 min. The tubes were placed in a rate freezing apparatus in the neck of a 35HC (Taylor-Wharton) liquid nitrogen dewar. The freezing rate was calibrated to be 0.5–1.0 K/min. The cells were maintained at 173 K until the NMR analysis.

The *t*-butanol-washed cells were prepared by resuspending the cells for 30 min at 298 K in a 2.5% solution of *t*-butanol in PBS. The cells were then washed three times with PBS and once with complete medium and frozen as described above.

The IMR-90 cells were carried in culture from population doubling level (PDL) 11.3, to assure a constant recording of the growth parameters. At PDL 45, which was calculated to represent 1 or 2 passages prior to morphological senescence, the cells were split into two groups. Group 1 was carried in culture with no change. Group 2 was treated with swainsonine (Boehringer Mannheim) at 1 μg/ml in 20 ml of growth media. The swainsonine was added to the culture medium and was present throughout the culture period. The cells were then treated as described above to prepare them for the NMR.

NMR measurements

Measurements of linewidth were made on a General Electric GN300 NMR spectrometer operating at a frequency of 300.1 MHz. Eight transients were summed to achieve sufficient signal to noise. Spectra were acquired using 1024 data points with approximately a 45° tip angle (5 μs pulsewidth) and a 8000 Hz sweepwidth. No apodization functions were used. The overlapping proton signals of the water and DMSO were deconvoluted using the GE software program GEMCAP. All samples were run unlocked.

Low temperatures were achieved by passing cold N_2 gas over the samples. The temperature was maintained with a GN-series temperature control unit, and control of ±0.1°C was maintained. The samples were maintained at temperature for 15 min prior to acquisition of data.

Statistical analysis was preformed using the program BIOSOFT from Elsevier Publications (Cambridge, U.K.).

Results

Fig. 1 shows a typical spectrum of a frozen cell suspension at 213 K. The signal from the unfrozen water protons at 4.8 ppm has a Lorentzian line shape, and the linewidth was determined using the GE program GEMCAP. As the temperature of the suspension is increased the water and DMSO resonances narrow and resolve.

Fig. 2 shows a plot of the linewidth versus temperature for IMR-90 cells at PDL 20 and at PDL 47. Standard error was determined from two different samples of IMR-90 PDL 29 cells and three different samples of IMR-90 PDL 47 cells. At all four different temperatures, the senescent IMR-90 cells have a smaller

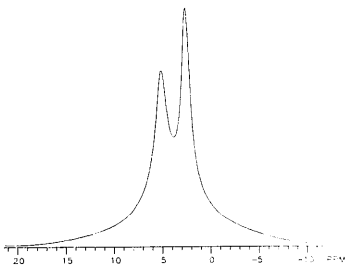

Fig. 1. Proton NMR spectrum at 300.1 MHz of frozen cell suspension in freezing medium containing 10% DMSO, at 213 K.

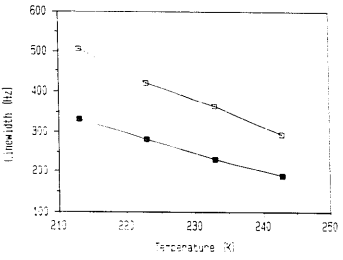

Fig. 3. Proton linewidth of the water in frozen cell suspensions versus temperature for IMR-90 cells PDL 41 before *t*-butanol wash (□), and after *t*-butanol wash (■).

linewidth than the IMR-90 cells at PDL 20. A comparison of the ratio of the intensities of the unfrozen water resonance to the DMSO resonance for the IMR-90 cells at PDL 29 and PDL 47 showed that the amount of unfrozen water remained unchanged, suggesting that the linewidth change did not result from a change in the amounts of cellular material. The linewidth values range from 936 Hz at 203 K to 219 Hz at 233 K. The results of a Student's *t*-test showed that the data, for the two cell types, at 203 K are not significant to the 5% level. The Student's *t*-test performed on the data from the other temperatures showed that the results are significant to at least the 5% level and at two temperatures (213 K and 233 K) the confidence level in the difference was greater than 1%.

Fig. 3 shows a plot of the linewidth versus temperature for IMR-90 cells at PDL 41 for two samples, one prepared in the normal manner and the other washed with *t*-butanol before freezing. At all four temperatures, the cells that were washed with *t*-butanol show a significant reduction in the observed linewidth of the water. The reduction in the linewidth is far greater than the experimental error determined for the data used in Fig. 2. Integration of the water peak area for the samples washed with *t*-butanol showed a 25% reduction in the amount of unfrozen water compared to a similar sample that was not washed with *t*-butanol. There is a difference in the water linewidth for the IMR-90 cells used in Fig. 2 with respect to that of the cells used in Fig. 3. This change is attributed to the difference in the PDL number of the IMR-90 cells, and may result from changes in the cell surface as the cells progress to senescence.

Fig. 4 shows a plot of the linewidth versus temperature for IMR-90 cells at PDL 47 with and without a treatment with swainsonine. The cells that were not treated with swainsonine display narrow lines as observed for senescent cells. Cells treated once with swainsonine display wider lines than the untreated cells. Swainsonine alters the cell surface by preventing the

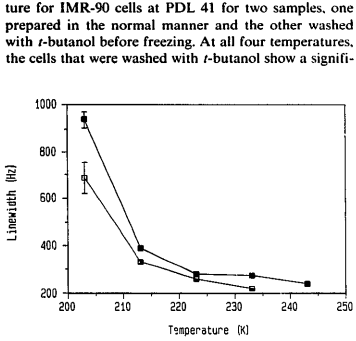

Fig. 2. Proton linewidth of the water in frozen cell suspensions versus temperature for IMR-90 cells: PDL 29 (■), PDL 47 (□).

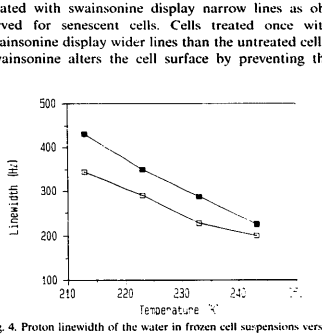

Fig. 4. Proton linewidth of the water in frozen cell suspensions versus temperature for IMR-90 cells PDL 47 with swainsonine treatment (■), and without swainsonine treatment (□).

TABLE I

Effect of t-butanol on cell surface oligosaccharide functional organization

Treatments	PDL	Morphological classification	K_{11}	K_{12}	R_1	R_2	Scatchard analysis ConA/MMP, *T* Anovar [a]
None	23	nonsenescent	1.06E06 ±2.1E05	2.22E03 ±5.0E03	2.17E−0.5 ±1.8E−06	3.09E−03 ±2.6E−04	
None	43	senescent		7.74E03 ±2.3E03		1.47E−03 ±1.0E−04	5.0105
t-BuOH [b]	20	nonsenescent		1836 ±92		1.02E−02 ±3.7E−03	5.0389
t-BuOH	43	senescent		1934 ±282		1.08E−02 ±1.4E−04	5.0384

[a] Student's *t*-test as part of *T* and analysis of variance. Groups all had six data points and the *t*-test compared the first set of data to the other three. The 1% significance level with 10 degrees of freedom is 3.17, thus all the values are highly significant.

[b] *t*-Butanol extraction, 2.5% *t*-butanol in PBS for 20 min at 298 K, followed by PBS washes and assessment of viability.

expression of complex carbohydrates on the cell surface. This change in the cell surface is manifested in the wider water line in the frozen cell suspension.

Table I shows data for experiments designed to assess the effect of *t*-butanol extraction on the ability of the cell surface oligosaccharides to interact with exogeneous lectins. The details of the methodologies used are given elsewhere [8–10]. The cells, either control or *t*-butanol extracted, were fixed, washed and blocked with PBS/BSA (0.1%). Biotinylated lectin (ConA from Vector) and various concentrations of synthetic competitor ligand (methyl α-D-mannopyranoside from Sigma) were mixed with the cells. After washing, further steps of avidin, biotinylated-alkaline-phosphatase and substrate were used to develop the assay. The optical density of the resulting solutions (405 nm) was transformed into a bound over free (B/F) parameter and plotted against the competitor concentration. Computer programs originally desinged by D. Rodbard (NIH) were used to analyze the data and choose the optimal statistical fit (1–5 parameters). The data show that cellular senescence and *t*-butanol extraction result in the apparent down-regulation of the cell surface, the loss of the high-affinity concanavalin A site while not significantly affecting the senescent cell population. These data are only a partial representation of the entire cell surface oligosaccharide display. Similar results were observed with the other lectins.

Discussion

The observed water signal in the frozen cell suspensions arises from the interaction of the water with macromolecules and other substances that disrupt the crystal lattice of the water. There are many contributions to the unfrozen water signal besides the water at the cell surface. These include water bound to other intracellular contents, and the DMSO present in the freezing mixture. The contribution of these substances to the linewidth is assumed to be constant from sample to sample so that any changes in the proton linewidth of the water is due to changes at the cell surface. The DMSO is present in the same quantity in all the samples. The intracellular contents have been shown to vary only slightly as the cells age even though the assembly rates of some cytoskeletal elements are altered [18]. The contribution to the signal from water at the glycocalyx will be large because of the greater surface area [19].

The *t*-butanol wash is known to remove small amounts of membrane bound proteins without damaging the cell [16]. When the cells were washed with *t*-butanol, the linewidth of the water signal narrowed considerably and integration showed an approximately a 25% decrease in the intensity of the signal from unfrozen water. The change in the intensity and linewidth of the unfrozen water could only result from alterations or reorganization of the oligosaccharides and bound proteins at the cell surface which results from an increase in the disorder of the local crystal lattice. The treatment of the cells with swainsonine is further evidence that changes in the cell surface are manifested as changes in the observed NMR water linewidth in frozen samples of cell suspensions. Swainsonine inhibits the Golgi enzyme α-mannosidase II and leads to the formation of hybrid-type oligosaccharides on the cell surface [18]. This reorganization of the oligosaccharides on the cell surface is observed as an increase in the NMR linewidth of the unfrozen water protons.

Table I shows a decrease in the binding affinity of ConA for oligosaccharide residues on the surface of IMR-90 cells as they age. A previous study [9] showed changes in the affinity of the lectin binding as well as changes in the mobility of the cell surface oligosaccharides were noted as IMR-90 cells aged. This observation

correlates with a narrowing of the proton linewidth noted for aged cells in the present study. If one neglects contributions to the linewidth due to field inhomogeneity, the linewidth is a complex function of the motional properties of the water molecule. As the correlation time for the molecular reorientations decreases the signal will broaden. In this study the motion of the water molecules becomes less hindered as the cells progress to senescence. This decrease in linewidth was observed by Kuntz et al. [14], for a loss of order and suggests that the change in the proton linewidth may be due to conformational changes in the cell surface oligosaccharides.

Conclusion

This study shows that the dynamics of cell surface oligosaccharides and bound proteins can be monitored though the properties of interacting water molecules, which are observed in rozen cell suspensions. The observed changes in the proton linewidth with senescence correlate with changes in surface structure observed in previous studies using lectin-based affinity assays [8–10]. It is thought that the changes in the oligosaccharides are either conformational changes or a loss of the oligosaccharides from the cell surface. Further studies are under way to pinpoint the exact mechanism by which the changes in the cell surface oligosaccharides are manifested by the properties of associated water.

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