Specific Detection of Glycans on a Plasma Membrane of Living Cells with Atomic Force Microscopy

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Summary

Among the many alterations of cancer cells is the expression of different surface oligosaccharides. In this work, oligosaccharide expression in living cells (cancer and reference ones) was studied with atomic force microscopy by using lectins as probes. The unbinding force obtained for the same lectin type (concanavalin A or *Sambucus nigra*) suggested slightly dissimilar structures of binding sites of the same ligand type. For the lectin from *Phaseolus vulgaris*, a much larger unbinding force indicated a distinct structure of the binding site in cancer cells. The unbinding probability confirmed a higher content of both sialic acid and mannose-containing ligands in cancer and reference cells, respectively. These results demonstrate the potential of atomic force microscopy to directly probe the presence of molecules on a living cell surface, together with the quantitative description of their expression.

Introduction

The majority of oligosaccharides present on the surface of a cell membrane form a glycocalyx. They are covalently linked to proteins or lipids (i.e., glycoproteins, glycolipids). Being their integral part, oligosaccharides become components of many important molecules such as structural and transport proteins, enzymes, immunoglobulins, and cell adhesion molecules [1]. It is known that these complex structures are in control of many processes, including cell embryonic development, cell differentiation, cell-cell interactions, and cell interactions with the extracellular matrix. On the other hand, they also contribute to the development of many serious diseases such as rheumatoid arthritis, viral and bacterial infections, and cancer [2, 3]. Cancer cells very often display distinct oligosaccharides that are supposed to play a critical role in tumor metastasis and escape from the immune response [4–7]. The alteration of oligosaccharides can encompass both changes in their abundance in cells and structural modifications. Some glycoproteins serving as tumor markers are also expressed in the normal counterparts, but the carbohydrate moiety of their glycans is often considerably different [8].

Many standard methods use lectins as probes with the aim of detecting carbohydrate structures. Lectins are proteins that recognize different types of oligosaccharides with very specific binding affinities comparable to those observed for enzyme-substrate or antigen-antibody interactions. They are commonly used in the characterization and isolation of simple and complex sugars [1], and they are also used as histological reagents in many areas of diagnostic investigation, especially those related to changes in the expression of cell membrane glycans [9–11]. The lectin from *Helix pomatia*, which binds to *N*-acetylgalactosamine residues of β1,6-branched glycans, has emerged as an interesting marker of cancer-altered glycosylation. Such alteration of the oligosaccharide expression has been reported for colorectal carcinoma, in which increased binding of the lectin from *Helix pomatia* was suggested to be a marker for metastasis [12]. The expression of glycoconjugates recognized by this lectin appears to be associated with a poor patient prognosis [13]. Sialic acid, recognized by the lectin from *Sambucus nigra* (SNA), determines the adhesive and antigenic properties (in cell to cell contact) that are altered during cell malignant transformation [14]. The lectin from *Phaseolus vulgaris* (PHA-L) was applied to study structural changes of *N*-linked oligosaccharides as a way of searching for a potential reactive glycoprotein that can be treated as an indicator of colon cancer. The results showed a carcinoembryonic antigen as the main target that can be further used as a tumor marker due to its overexpression in this and a number of other types of cancer [15].

During past decades, atomic force microscopy (AFM) was widely used in the study of the structure and function of a variety of biological systems in a liquid environment, close to the natural one [16–18]. Much effort was made in order to obtain high-resolution images of DNA [19], three-dimensional structures of proteins [20], and the topography of surfaces of both living and fixed cells [21, 22]. A great advantage of AFM is its ability to measure the sample stiffness attributed to the state of the cytoskeleton structure of living cells [23, 24] or to directly measure the force required to separate a pair of interacting molecules [25]. It is the latter measurement that, if performed on living cells in nearly natural conditions, provides new insight into the mechanism of biological interactions. Such studies are typically focused on a model system made up of pure components and characterized by high-affinity interactions, particularly those between biotin and avidin [26]. Recently, they have been extended to a wide range of receptor-ligand pairs as antigens and antibodies [27, 28] or SNARE

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(soluble N-ethyl-maleimide-sensitive factor attachment protein receptor) complex [29].

The lectin-oligosaccharide recognition process was also studied by AFM, but it was restricted to the lectin receptors immobilized on a hard substrate [30]. Only a few attempts were made to analyze the strength of the lectin-oligosaccharide interaction measured on a surface of living cells. In the case of concanavalin A and mannose-bearing ligands present on the walls of yeast cells [31], the range of the interaction forces was estimated (from 75 pN to 200 pN), while an exact force value was determined from the force distribution (116 ± 17 pN) obtained for concanavalin A and ligands containing mannose present on a surface of human prostate carcinoma cells [32]. Another example of the lectin-oligosaccharide interaction is the molecular complex formed between the lectin from *Helix pomatia* and N-acetylgalactosamine-terminated glycolipids of erythrocytes of group A. With the AFM probe modified by lectin, it was possible to distinguish between the erythrocytes with group A and those with group 0 within a given population of blood cells [33].

In this work, the plasma membrane oligosaccharides of two human bladder cell lines were probed directly on a surface of living cell by using atomic force microscopy. The probing tip was functionalized with three distinct lectins: concanavalin A (ConA), lectins from *Sambucus nigra* (SNA), and lectins from *Phaseolus vulgaris* (PHA-L). The measurements were performed in order to quantify the expression of oligosaccharides on the plasma membranes of bladder cancer cells. This was accomplished via the unbinding force, giving the strength of interaction within a single pair of molecules, and by the unbinding probability, indicating the number of oligosaccharide ligands present on a surface of living bladder cells. The obtained results showed differences of the oligosaccharide expression between cancer cells (T24) and the reference cells (HCV29). Both the number of given glycan types and the distinct character of binding (i.e., the formation of weak or strong bonds described by small or large unbinding force) were observed to be altered upon cancer transformation.

Results

Force Curves Selection

The direct interaction force between chosen lectins (ConA, PHA-L, and SNA) and their oligosaccharide ligands present on the plasma membrane of living cells was determined for both reference (HCV29) and cancer human bladder transitional cells (T24).

A typical force curve with one unbinding event, i.e., dependence of cantilever deflection on relative sample position, is presented in Figure 1A. Its main character was independent of the cells (T24 versus HCV29) and lectins (ConA, SNA, PHA-L) used. The measurements performed on a living cell membrane with a bare silicon nitride cantilever showed neither unbinding events nor nonspecific adhesion forces Figure 1B. All force curves with a characteristic jump (Figure 1C, probably indicating a destructive punching of the cell membrane) and those with multiple unbinding events (Figure 1D, suggesting the potential influence of other molecules) were omitted from analysis.

The Unbinding Force Histogram

The force distributions measured between lectins and the surface oligosaccharides of human bladder cells (HCV29 and T24) are presented in Figure 2. The histograms were created with the bin size of 15 pN. This value corresponds to the minimum detected force, estimated from the equation: $F_{det} = (k_B \cdot T \cdot k)^{0.5}$, where k is the cantilever spring constant, k_B is the Boltzmann constant, and T is temperature. For a cantilever with the spring constant of 0.03 N/m, in room temperature the minimum detected force is 11.5 pN. This minimum detected force was also independently determined from the noise fluctuations of the baseline of force curves (F_{det} = 13.1 ± 3.2 pN).

The interaction forces between molecules have two components: (1) a discrete component dominating within the binding sites (short-range), which is related to the bond strength of the single molecular pair, and (2) a component originating from long-range distance-dependent forces, dominating outside of the binding site. Since determination of the molecular interaction forces is based on the measurements of a force needed to separate an AFM tip from the investigated surface, in principle it is possible to detach oligosaccharide moieties from their receptor proteins. Such an event seems extremely unlikely, though, due to the relatively strong covalent bonds keeping the two together. A rough conversion of the binding energy to the binding force for different types of bonds (covalent, etc.) was performed by taking into account the potential energy of a spring ($E = 0.5 \cdot k \cdot x^2$) and the force ($F = k \cdot x$) acting on a distance equal to the bond length (i.e., 0.15 nm for S–S). In this way, a covalent bond was estimated to be stronger than 5 nN. In the measurements presented here, the interaction force was never this large. It did not exceed the value of 1 nN, which corresponded to weaker interaction forces, such as ionic ($\sim$0.44 nN), van der Waals ($\sim$0.04 nN), or hydrogen bonds (ranging from 0.14 to 0.88 nN). The determined interaction strength of the studied lectin-oligosaccharide complexes ranged from 50 pN to 200 pN regardless of the cell and lectin type. The figures were comparable with typical unbinding force values measured by using AFM for antigen-antibody interactions [27, 28].

The maximum value of the measured force did not exceed 0.4 nN, except in the case of the T24 cell surfaces probed with the PHA-L-coated cantilever when the observed maximum force was $\sim$0.8 nN. All histograms showed that more then one force peak was observed within the contact area between the functionalized AFM probe and the cell surface (except when the ConA-modified probe was scanned over the surface of HCV29 cells). By fitting the Gauss function, the values of the unbinding force for each histogram were determined. The unbinding force, *F*, is a center of a Gaussian fit, and the corresponding error is a standard deviation determined from its half width.

The observed peaks (marked by stars in Figure 2) were attributed to the formation of subsequent bonds within the contact area of the AFM probe and cell surface. The unbinding probability was always below 30%, regardless of the studied case, indicating that only a few bonds could be created within the contact area [34]. The observed multiple peaks in force histograms indicated simultaneous rupture of the subsequent bonds.
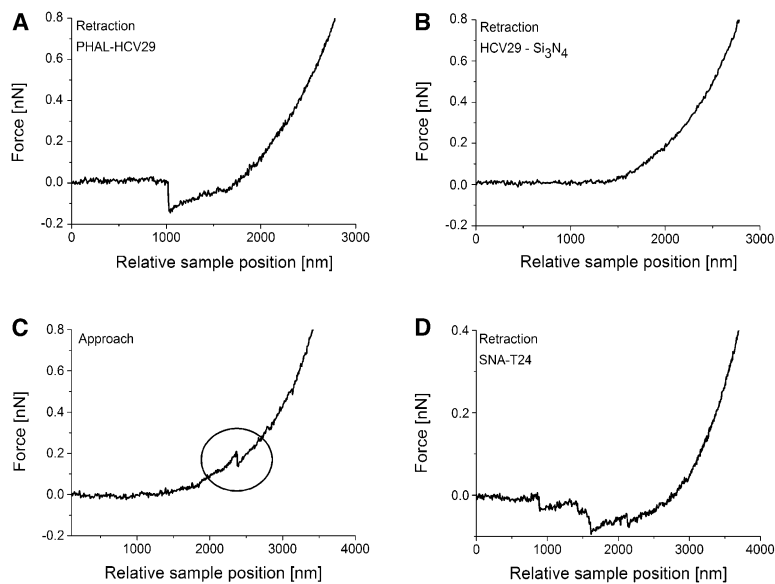
Figure 1. Force Curve Recorded on the Surface of Living Cells

(A) An HCV29 cell probed with a PHA-L-functionalized silicon nitride tip.

(B–D) An HCV29 cell surface probed with a bare silicon nitride tip, with (C) a jump indicating the punching of the cell membrane, and (D) multiple adhesion events observed for an SNA-covered AFM tip and the surface of T24 cells.

When the surface of the reference HCV29 cells was probed by using the AFM cantilever modified with concanavalin A, only one peak was observed. This could mean that there was only one type of mannose-bearing oligosaccharide capable of binding to the lectin in the contact area ($\sim 0.3\ \mu m^2$). In contrast to reference cells, the cancer cells showed four peaks (where each peak corresponded to subsequent rupture of one, two, three, or four bonds at the same moment), suggesting that more than one type of mannose-bearing ligand could interact with ConA. Since the area under the peaks is related to the probability of a given number of accessible ligands, simultaneous unbinding of two or more bonds between ConA and its ligands was less probable than unbinding of one bond. A similar effect was observed for the lectin from *Sambucus nigra*, in which three and four peaks were detected for reference and cancer cells, respectively. The most unexpected result was obtained when the surface of T24 and HVC29 cells was probed with the AFM cantilever coated with PHA-L. The number
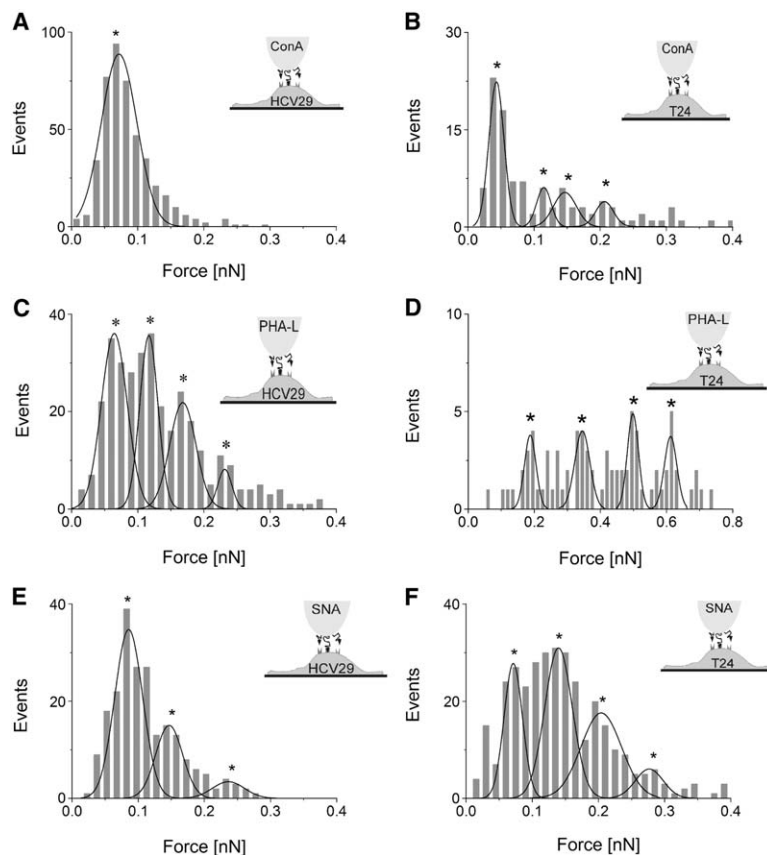

Figure 2. Force Histograms Obtained for Bladder Cells Probed with Lectins

(A–F) (A, C, and E) HCV29 and (B, D, and F) T24 cells were probed with three types of lectins—ConA, SNA, and PHA-L. The bin size was 15 pN, and it was determined from the noise fluctuations of the baseline of force curves. The multiple peaks are marked by stars. The fitted Gaussian function (line) was employed to determine the unbinding forces.

of unbinding events was larger for the reference cells, and similarl to what is seen with other lectins, the probability of breaking bonds was the same for the formation of one or two bonds. For cancer cells, the force histogram showed four maxima corresponding to the rupture of the consecutive bonds. Their almost equal area indicated that *N*-acetylglucosamine residues of β1,6-branched glycans were more accessible for PHA-L than those present in reference cells.

Unbinding Force of a Single Molecular Complex

A common way of determining the unbinding force for a single molecular pair attributes the position of the first peak in the obtained histogram to the unbinding event of a single molecular complex, assuming that the unbinding probability is lower than 30% [34]. In that case, only a few molecular bonds are expected to be formed. Thus, the presence of multiple peaks in force histograms can be explained as follows. The first peak corresponds to the unbinding event involving the rupture of one bond, the second is related to the simultaneous unbinding of two bonds (the force values at the second maximum are doubled), the third is related to the unbinding of three bonds, etc. Such a force histogram can be translated into the relationship of the unbinding force determined for each consecutive peak and the peak number (i.e., bond number); moreover, linear dependence is expected if only one type of interaction is present. A superposition of a few types of interactions caused the experimental data to deviate from the fitted line.

Figure 3 presents such a linear relationship for all investigated cases except ConA-HCV29, in which only one peak was present in the force histogram (Figure 3A). An exact value of the unbinding force for a given individual lectin-oligosaccharide complex was determined from the slope of the fitted line (all results are summarized in Table 1). Insets in Figure 3A show the comparison of the unbinding force determined for the single pair corresponding to (i) mannose-ConA, (ii) N-acetylglucosamine-PHA-L, and (iii) sialic acids-SNA interactions studied on the surface of living HCV29 and T24 cells. The obtained unbinding force of a single complex overlapped within the experimental error with the values determined on the basis of the unbinding force histogram. Only in the case of AFM cantilevers modified with PHA-L and its oligosaccharide ligand present on a surface of T24 cells were the values of the unbinding force obtained from the linear regression significantly smaller than those estimated from the histogram. This can be explained by the presence of nonspecific forces that compete with the specific ones. Such non-specific forces can arise from the procedure of the cantilever modification (PHA-L was directly attached to the surface of the AFM probe without any spacer). The advantage of using linear regression is that errors are smaller and the obtained value is only slightly influenced by other non-specific forces.

In order to statistically evaluate the obtained differences between cell lines for each fitted line, the confidence bands of 95% were calculated. They estimate the certainty of the shape of the fitted line, and the assumed confidence level implies a 95% chance that the true regression line fits within these bands. This approach works reasonably well when the regression
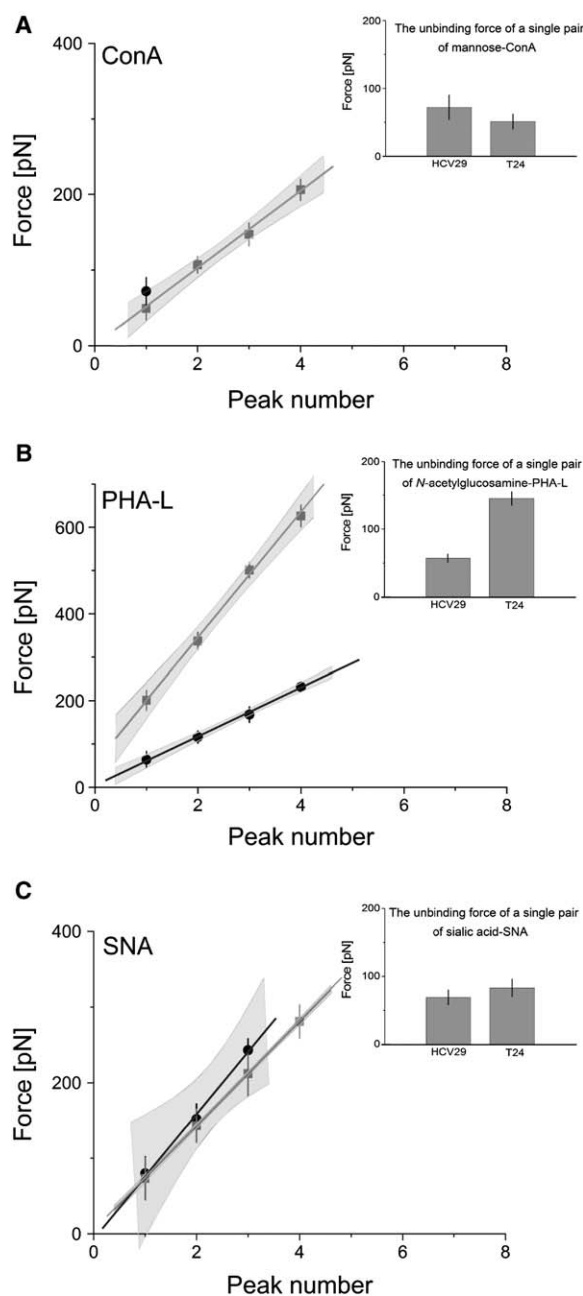

Figure 3. Linear Regression Fitted to the Unbinding Force as a Function of the Number of Succeeding Peaks Denoting the Presence of Only One Type of Interaction

(A–C) The surface of HCV29 (black line and dots) and T24 (gray line and dots) cells was probed with three types of lectins—ConA, SNA, and PHA-L. Data points correspond to centers of Gaussians fitted to each single maximum from Figure 2, while error bars represent their standard deviations. The correlation factors for all studied cases ranged from 0.998 to 0.999. For each curve, confidence bands (95%) were calculated. They estimate the certainty of the shape of the fitted line. The assumed confidence band implies a 95% chance that the true regression line fits within these bands. Insets: the comparison of the unbinding force determined for the single pair corresponding to (A) mannose-ConA, (B) N-acetylglucosamine-PHA-L, and (C) sialic acids-SNA interactions studied on the surface of living HCV29 and T24 cells.

Table 1. The Comparison of the Two Approaches of the Unbinding Force Determination

Cell Lines	Lectin Type	F [pN] (Linear Regression)	F [pN] (Force Histogram)
HCV29	ConA	–	72 ± 18
	PHA-L	57 ± 6	64 ± 19
	SNA	83 ± 13	80 ± 22
T24	ConA	51 ± 11	49 ± 15
	PHA-L	145 ± 10	201 ± 23
	SNA	69 ± 11	73 ± 28

The following complexes were measured: (1) N-acetylglucosamine-PHA-L, (2) mannose-ConA, and (3) sialic acid-SNA. The value of the unbinding force was determined by two approaches: (1) by linear regression (see Figure 3), in which the force value and its error correspond to the slope and the standard deviation of the slope, respectively, and (2) by the first force peak present in force histograms, in which the error is a half width of the peak at half height and denotes the standard deviation.

curve is calculated based on more points, which, in our studies, corresponds to cases in which histograms were composed of four maxima. However, for the case in which only three maxima were observed (Figure 3C, the HCV29 cell surface was probed with the lectin SNA in search of sialic acids residues), this method produces a rather large confidence range that illustrates the limitations of this approach.

Unbinding Probability

The unbinding probability is usually defined as the ratio of the number of unbinding events to the total number of recorded force curves. Its value corresponds to the number of bonds that can be formed under given experimental conditions. Figure 4 presents a comparison of the unbinding probability performed for HCV29 and T24 cells. The surface glycans containing either sialic acid at various glycans or multiple mannoses at mostly high-mannose glycans, or N-acetylglucosamine residues at β1,6-branched glycans, were probed with a given type of lectin (SNA, ConA, PHA-L). The amount of sialic acids residues recognized by lectin from Sambucus nigra increased in cancer cells (from 5.3% to 8.4%). The number of mannose and N-acetylglucosamine residues bearing glycans decreased dramatically
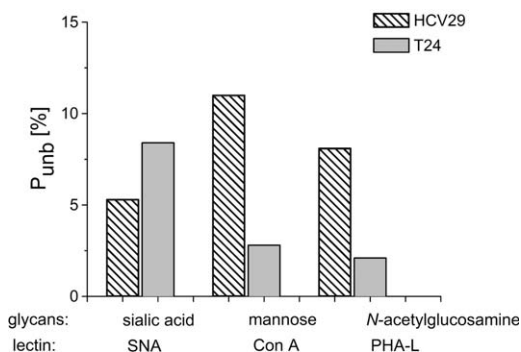

Figure 4. Unbinding Probability of Cell Surface Glycans

The unbinding probability determined for cell surface glycans with the use of three types of lectins—ConA, SNA, and PHA-L—showed the differences of their expression in cancer (T24) and reference (HCV29) cells.
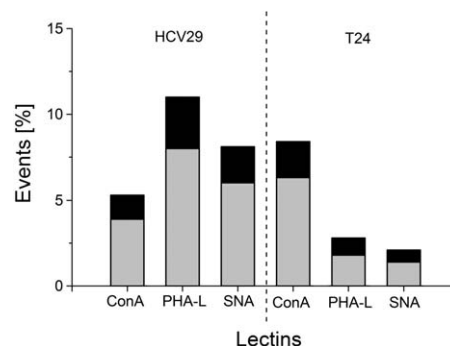

Figure 5. Binding Site Inhibition

The observed decrease of the number of unbinding events after (gray columns) inhibition of the interaction with cell surface glycans compared to control measurements (before, black columns) verified the specificity of the studied interactions.

from 11% to 2.8% and 8.1% to 2.1%, respectively, indicating their lower expression in cancer T24 cells.

Binding Sites Inhibition

To show that the measured unbinding events originated from the specific interaction of the lectin-glycan complexes, the access to potential glycan binding sites was blocked by adding a given type of the studied lectin (50 μg/ml) to TBS buffer for 30 min of incubation. Next, cells were washed with TBS buffer only to remove excess unbound lectins, and cells were immediately measured with AFM by using the lectin-modified AFM cantilever (the same type as that used for inhibition). In all cases, the number of the recorded force curves showing unbinding events decreased, confirming the specificity of the studied interaction (Figure 5).

Discussion

It has been reported for many tumors that the plasma membrane oligosaccharides linked to proteins or lipids are altered during cancer transformation. This fact influences tumor cell invasion and adhesion, leading to disturbances in the cell's interaction with both the extracellular matrix and the neighboring cells [35–39]. Therefore, changes in the expression and in the structure of oligosaccharides seem to be a characteristic feature of the malignant transformation [40]. Here, the atomic force microscopy technique was applied to quantitative characterization of the expression of oligosaccharides by using two factors: the unbinding force and the unbinding probability.

The unbinding force, directly determined from AFM measurements, denotes the strength of the interaction that makes up a single pair of molecules. When a given adhesion molecule is probed with the same ligand, the unbinding force is expected to be the same if the structures of both the adhesion molecule and the ligand are unchanged. Force variations can indicate changes in the structure of the binding site either in the adhesion molecule or in the ligand. The unbinding forces determined for oligosaccharides probed by the same lectin type (cf. Table 1) were expected to be the same if the structure of the ligand binding site was unchanged upon cancer transformation. The obtained results

showed a small dissimilarity in the unbinding force values for ConA and SNA lectins, indicating, in both cases, a somewhat altered structure of the binding site of the cell surface ligands. A distinct difference was obtained for PHA-L, in which the unbinding force measured for reference cells was about 2.5 times lower than that of cancer cells. This fact can be attributed to significant structural changes of oligosaccharide ligands caused by cancer transformation influencing the binding stability. On the other hand, the unbinding force value characterizes the interaction as weak or strong, which corresponds to the formation of a more or less stable complex, respectively. The higher values thus indicated the formation of more stable complexes with oligosaccharides (composed of mannose and sialic acids, respectively) present in nonmalignant cells (HCV29) than with those present on the cancer surface (T24 cells).

The unbinding probability can be attributed to the number of single bonds that are formed between the lectin and its ligand present on a cell surface; in a quantitative way, the unbinding probability describes the expression of cell surface molecules. The values determined by AFM in this work were in agreement with indirect (and rather qualitative) results obtained by means of standard biochemical methods [41]. Cancerous T24 cells appear to be richer in sialic acid residues than the reference HCV29 cells (8.4% versus 5.3%, respectively) due to the reported [42] increased sialylation of cancer cells. The increased expression of mannose-bearing ligands (11%) in HCV29 cells as compared with that of mannose-bearing ligands in cancer T24 cells is an indication of a much higher amount of the high-mannose-type glycans on their surface and was previously reported by Przybyło et al. [41] for the same cell line. A specific interaction with PHA-L lectin indicated the presence of triantennary or tetraantennary structures (GlcNac β1,6-branched) of complex-type glycans [43]. A decrease of the unbinding probability of PHA-L (from 8.1% to 2.1%) suggested the loss of such structures in T24 cancer cells.

Owing to the constant contact area maintained between the probing tip and the cell surface, and its very high force and spatial resolutions, AFM can measure multiple unbinding events if only their formation is possible. All studied lectins at a pH above 7.0 are in a tetramer form and potentially have four identical binding sites (ConA [43], PHA-L [44], and SNA [45]). Steric organization of the plasma membrane oligosaccharides at the cell or molecule surface is very significant in view of their accessibility to the lectin. Therefore, multiple peaks observed in the unbinding force histograms can be attributed either to the number of lectin binding sites that are involved in the oligosaccharide recognition process or to the number of ligands present on a cell surface in the form of a group composed of a few closely located single oligosaccharide chains involved in the adhesive interaction with the lectin [46]. The analysis of force histograms showed that such "clustering" of cell surface glycans was most pronounced for PHA-L and SNA lectins. In the case of ConA measurements performed on the surface of reference cells, it indicated only one bond formed between ConA and its mannose ligands on the cell surface. In general, however, clusters of glycans were more likely to appear in reference cells

than in cancerous ones. This follows from the number of recorded unbinding events.

Despite the fact that AFM has become a method that is widely applied to the study of biology-related problems, its advantages over standard biochemical methods are still not fully recognized. Some of them are obvious; for example, there is no need to use fluorescent-labeled lectins (with the exception of very specific cases). Moreover, in the AFM study, the native protein is immobilized by means of a fairly uncomplicated protocol. By using fluorescence-based methods, the detection of lectin binding sites can be performed relatively easily, but gathering quantitative information about their number (or density) and about the strength of the interaction is a bit more difficult. The use of immunodetection of blotted proteins shows the presence of lectin binding as a band of a given molecular mass of the formed complexes. However, these experiments deliver only qualitative information. Furthermore, in these methods, the cell membrane should be first blocked to prevent nonspecific antibody binding. This step is not needed in AFM measurements since the specific recognition events manifest either as a single peak with a given force value (HCV29-ConA) or as a periodicity in the force histogram (as in the HCV29-SNA complex).

Summarizing, in AFM experiments, one type of molecule (i.e., lectins) is immobilized on the AFM probe, and the other, complementary one should be present on the investigated surface (either it is an isolated protein deposited on a given support or the cell membrane). This technique delivers the possibility of obtaining a detailed and quantitative description of the expression of molecules present on a surface of living cells. By using AFM, one can determine (1) the strength of the interaction of a given molecular complex, reflecting the bond stability, and (2) the unbinding probability corresponding to the number of molecules present on the cell surface. Both parameters can be used to trace the changes (induced, e.g., by cancer transformation) of a given pair of molecules, and additional information about changes in the number of cell surface molecules or about alterations occurring within the binding site, for example, can be gathered.

Significance

The characterization of differences in the oligosaccharide expression on a surface of two living bladder cell lines (reference HCV29 and cancerous T24) was performed by using atomic force microscopy (AFM). The oligosaccharide expression was quantitatively described by two parameters. The first one is the unbinding force of a single pair of molecules. It is directly measured by AFM with very high-force resolution down to tens of piconewtons, and its value is characteristic for a studied complex under the given experimental conditions. A stronger unbinding force means a more difficult rupture of the molecular complex and thereby indicates that a more stable complex has been formed. When a certain ligand type, recognizing the specific structural fragment of its receptor, is immobilized on the AFM probe, any variation of the force value indicates an alteration in the binding site structure of the receptor. The second parameter, the

unbinding probability, corresponds to the number of molecules that are present on a surface of living cells and can be related to the density of surface receptors. The obtained results showed distinct expression of plasma membrane oligosaccharides for cancerous cells (T24), which suggests the use of AFM as an alternative technique with respect to other biochemical methods. AFM allows for quantitative characterization of the expression of any type of surface molecule by direct measurement of the interaction strength within a single pair of molecules (i.e., the unbinding force). These results also highlight the applicability of AFM for investigating the interaction forces between single molecules in the field of cancer drug development.

Experimental Procedures

Cell Lines
The studies were carried out on human bladder epithelial cells: HCV29 (nonmalignant transitional epithelial cells of the ureter) and T24 (transitional cancer cells of the urine bladder). The cells were cultured on Petri dishes in OptiMEM medium (GIBCO-BRL [pH 7.4]) at 37ºC in a 95% air/5% CO_2 atmosphere. Next, after the same number of passages, they were cultured on a glass coverslip pretreated with poly-L-lysine (0.01% v/v) in order to improve their adhesion to the glass substrate [47].

Lectins
Three different types of lectins were used: ConA (concanavalin A from *Canavalia ensiformis*, ICN Biomedicals, Inc.), PHA-L *(Phaseolus vulgaris leucoagglutinin*, ICN Biomedicals, Inc.), and SNA (*Sambucus nigra*, Sigma-Aldrich). Their oligosaccharide ligands are mannose (or glucose), *N*-acetylglucosamine, and sialic acid in appropriate structural contexts, respectively. Each lectin was dissolved in 10 mM phosphate-buffered saline (PBS [pH 7.4], Sigma-Aldrich) at the final concentration of 0.1 mg/ml.

Cantilever Functionalization with Lectins
The lectin immobilization procedure was done as described in [32]. Briefly, standard silicon nitride cantilevers (MLCT-AUHW, Veeco) were cleaned by immersing them in acetone for 5 min and then irradiating them with UV light for 30 min. Then, after silanization with 3-aminopropyltriethoxysilane (APTES; Sigma-Aldrich), they were immersed in glutaraldehyde (Fluka) dissolved in PBS (2.5% [v/v]) for 30 min. Afterwards, they were washed with PBS buffer and functionalized by sitting in lectin solution (0.1 mg/ml) for 1 hr.

The applied protocols of protein immobilization onto the AFM tip enrich its surface with randomly oriented molecules. However, within the large number of molecules attached, there is always a fraction that can interact with oligosaccharide ligands present on a cell surface [48].

AFM Measurements
Commercially available silicon nitride cantilevers with the spring constant of 0.03 N/m (MLCT-AUHW, Veeco) and a nominal tip radius of about 50 nm were used as a probe. Their spring constants were controlled by monitoring the resonant frequency of the thermally excited cantilever. The tip radii were determined by using the TGT01 silicon standard (NMDT), and their values were about 60 nm for nonfunctionalized tips. Lectins were attached to the AFM tip, as it was described earlier [32], causing the tip radius to increase to about 100 nm.

A home-built AFM [47, 49] equipped with a "liquid cell" setup was used. To provide native conditions and to assure lectin binding activity, measurements were done in Tris-buffered saline (TBS = 50 mM TRIS-HCl + 150 mM NaCl [pH = 7.4]) supplemented with Ca^{2+}, Mg^{2+}, and Mn^{2+} ions at 1 mM concentration. The force curves were recorded close to a cell center. They were repeated 4–6 times by using a fresh AFM tip (with newly immobilized lectin) and a new sample (fresh cultured cells on a glass substrate). All measurements were carried out at room temperature.

Force Curves
The force curves, measurements of the dependence of the cantilever deflection on the relative sample position, were recorded for the same retraction velocity of 780 nm/s. In order to obtain the distribution of the binding sites on the cell surface, force curves were recorded over a certain randomly selected area of the cell surface. Typically, the recorded pattern contained squares of 11 × 11 points that corresponded to a surface area of 10.7 μm × 10.7 μm. The distance between the two points in the AFM interaction map was ∼1 μm. The true contact area between the AFM tip and the cell surface was estimated on the basis of Hertz mechanics for the radius of the tip curvature of 100 nm. The estimated radius of the contact area was around 250–300 nm for both cell lines, assuming the Young's modulus of 7.5 (±3.6) kPa and 1.0 (±0.6) kPa for HCV29 and T24 cells, respectively [49]. To avoid the possibility of measuring the same (or partially the same) spot more than once, the maximum loading force was limited so that the real contact area was smaller than 1 μm^2 per one spot; it was below 2 nN.

In order to collect the information about the alterations present in an entire cell population, a few coverslips with cells were measured (4–6). The total number of cells measured (force curves analyzed) was 33 (3982), 36 (4267), 34 (4057), 33 (3983), 31 (3715), and 34 (4095) for the interaction occurring between HCV29-ConA, HCV29-PHA-L, HCV29-SNA, T24-ConA, T24-PHA-L, and T24-SNA, respectively.

Data Verification
In order to assure that the force measured by AFM originated from the specific interaction between lectins and their glycans present on the cell plasma membrane, the surface lectins' binding sites were blocked during the course of a 30 min incubation of cells with TBS buffer containing 50 μg/ml of a given lectin type (ConA, PHA-L, SNA). Next, the cells were washed with TBS buffer (only to remove unbound lectins) and immediately measured with AFM by using the lectin-modified AFM cantilever (the same type as that used for inhibition).

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