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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 (~0.44 nN), van der Waals (~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 ~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.