Altered glycan expression on breast cancer cells facilitates infection by T3 serotype oncolytic reovirus

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ABSTRACT: Breast cancer is the most common cancer in women. Although current therapies have increased survival rates for some breast cancer types, other aggressive invasive breast cancers remain difficult to treat. As the onset of breast cancer is often associated with the appearance of extracellular markers, these could be used to better target therapeutic agents. Here, we demonstrated by nanobiophysical approaches that overexpression of α-sialylated glycans in breast cancer provides an opportunity to combat cancer cells with oncolytic reoviruses. Notably, a correlation between cellular glycan expression and the mechanical properties of reovirus attachment and infection is observed in a serotype-dependent manner. Furthermore, we enhance the infectivity of reoviruses in malignant cells by the coinjection of α-sialylated glycans. In conclusion, this study supports both the use of reoviruses as an oncolytic agent in nanomedicine and the role of α-sialylated glycans as adjuvants in oncolysis, offering new perspective in oncolytic cancer therapy.

KEYWORDS: Atomic force microscopy, cell mechanics, reovirus, breast cancer cells, sialylated glycans

Altered glycosylation is a universal feature of cancer cells. Tumor cells undergo activation and rapid growth, which are often accompanied by changes in cellular glycosylation profiles, as observed with normal cells during embryogenesis. Some types of glycans, known as tumor-associated carbohydrate antigens (TACAs), are markers of tumor progression and serve as specific targets for therapeutic intervention. Sialylation of proteins, lipids, and carbohydrates, an example of glycosylation, plays an important role in cell recognition, adhesion, and signaling. An increase in sialylation, especially with α2,3- and α2,6-linked sialic acid (SA), occurs in breast cancer. Despite the important role of glycosylation during cancer progression, there are only limited avenues to target altered glycosylation profiles for therapy.

Reovirus forms icosahedral particles (~85 nm) that contain a segmented double-stranded RNA genome enclosed by concentric protein shells called outer capsid and core. The core consists of the dsRNA genome, core proteins, and the β2 protein, which spans the core and outer capsid. The σ1 protein is a trimeric fiber that has discrete regions that mediate binding to cell-surface receptors. Sequences in the C-terminal σ1 head domain of serotype 1 (T1) reoviruses bind α2,3-sialylated glycans present on proteins and the GM2 ganglioside, whereas a domain in the tail of serotype 3 (T3) reovirus σ1 binds α2,6-sialylated glycans terminating in SA (NeuSAC) and α2,3-sialylated glycans. For both serotypes, the σ1 head also binds the proteinaceous receptor, junctional adhesion molecule-A (JAM-A) (Figure 1b, gray). Incubation of T3 reovirus with soluble α-linked SA strengthens the interaction between virions and JAM-A, likely by inducing a conformational change in the T3 σ1 protein.

Reovirus oncolytic properties were discovered in the late 1970s with additional work showing that reovirus is capable of efficiently infecting cells with an activated Ras signaling pathway relative to untransformed cells. These findings are important, as activating mutations in Ras genes alone contribute to more than 30% of all human cancers. In this context, reovirus is a promising candidate for development of new therapeutics for a wide array of cancers, including triple-negative breast cancer (TNBC). Current efforts focus on increasing the intrinsic capacity of reovirus to target and kill highly malignant cells, optimizing the efficacy of reovirus combination therapies, and assessing the effect of reovirus on immunotherapy. Essential for each of these efforts is an understanding of how different reovirus serotypes bind and infect cells that display different degrees of cancer progression with differential glycan expression. Atomic force microscopy...
(AFM) allows simultaneous imaging and probing of biochemical and mechanical properties of biological systems under physiological conditions. Using an AFM tip appended with reovirus virions, mechanical properties of interactions between virions and cells can be quantified to biophysically characterize the first step in virus attachment to the cell surface to initiate infection. Here, we used MCF-10A, MCF-7, and MDA-MB-231 cell lines as a model of breast cancer progression, that are nonmalignant, mildly malignant, and malignant metastatic human breast cells, respectively. Using AFM, we first extracted their mechanical properties and confirmed a significant modification of the Young’s modulus in good agreement with the degree of malignancy of the cell line. Next, AFM tips functionalized with two reovirus strains were used to probe the interaction to two different SA glycans (GM2 and GM3) on model surfaces, and we observed strong differences among serotypes. On living cells, we observed that only T3SA+ reovirus, the strain capable of engaging GM3, binds preferentially to breast cancer malignant cells, making it a stronger candidate for therapeutic applications. In addition, T3SA+ binding properties to the cell surface correlate with cellular

Figure 1. Probing reovirus binding to glycan model surfaces. (a) Schematic representation of reovirus particles with labeled outer-capsid proteins. (b) Schematic representation of reovirus σ1 for T1L and T3D. The glycan-binding sites of serotypes 1 (T1) and 3 (T3) are located in different domains of σ1. T1L reovirus binds to the GM2 glycan while T3D binds to the GM3 glycan. The T1 and T3 σ1 head domains also bind to JAM-A. (c) Schematic representation of the AFM cantilever functionalized with a reovirus virion. The reovirus-bound AFM tip scans the model surface of GM2 or GM3 glycan and binding events are recorded. (d) Schematic of force versus distance curve (FD curve) from which the binding force is extracted as well as the binding frequency (as the ratio between the number of FD curves with specific adhesion events and the total number of FD recorded). (e) The BE model describing a virus-glycan bond as a two-state model. The bound state is separated from the unbound state by a single energy barrier located at distance $x_u$. $k_{off}$ represents the dissociation rate. (f) Binding frequency of T1SA+/T1SA- and T3SA+/T3SA- on GM2 and GM3 model surfaces. T3SA+ has a higher affinity for GM3, and T1SA+ has a higher affinity for GM2. Each dot represents the binding frequency extracted from one map, consisting of 1024 FD curves. (g,h) Dynamic force spectroscopy (DFS) plot showing the distribution of rupture forces measured between T1SA+ and the GM2 surface (g) and T3SA+ and the GM3 surface (h). Each data point corresponds to the force extracted from an individual FD curve. Data corresponding to single interactions are fitted with the BE model describing a ligand–receptor bond as a simple two-state model (I, black curve). Dashed lines represent the predicted binding forces for two (II) and three (III) simultaneous uncorrelated interactions (Williams-Evans model [WEM]). Error bars indicate SD of the mean value. Distributions were evaluated using one-way ANOVA followed by posthoc Tukey’s HSD tests. For all experiments, data are representative of at least $n=3$ independent experiments. **** $P<0.0001$. 

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infectivity, showing an increased infectivity of malignant cells. Strikingly, we demonstrate that viral infection efficiency can be enhanced by the addition of short soluble α-SA glycans. These findings open new avenues for breast cancer nanomedicine and pave the way to further improve the use of reoviruses as oncolytic agents.

**RESULTS**

Reovirus Binds Sialylated Glycans in a Serotype-Specific Manner. As binding to SA serves as the first foothold on the cell surface for reovirus, we used AFM to evaluate the binding strength of reovirus serotypes to specific sialylated glycans. These findings open new avenues for breast cancer nanomedicine and pave the way to further improve the use of reoviruses as oncolytic agents.

Figure 2. T3SA+ binding increases on malignant cells. (a) An AFM tip functionalized with T3SA+ and T3SA- reovirus virions is cyclically approached and retracted from living cells. (b) From the AFM cantilever deflection, a force versus distance (F−D) curve is constructed allowing the Young’s modulus and adhesion force to be recorded. (c) AFM height images and corresponding (d) elasticity and (e) adhesion maps for MCF-10A (nonmalignant), MCF-7 (mildly malignant), and MDA-MB-231 (malignant metastatic) breast epithelial cells. (f) Young’s modulus of the cell decreases with the degree of malignancy. Each data point represents the mean Young’s modulus value extracted from a single cell. (g) Binding frequency extracted from adhesion maps for MCF-10A, MCF-7, and MDA-MB-231 cells. Binding probability significantly decreases on the three cell types for the T3SA-. We also observe a higher binding frequency for the both malignant cells with T3SA+. The error bars indicate SD with $n=7−29$ for each condition. Distributions in panels f and g were evaluated using one-way ANOVA followed by post hoc Tukey’s HSD tests. *$P<0.05$, ****$P<0.001$, and n.s. nonsignificant.
+/T1SA- and T3SA+/T3SA-, which are isogenic, with the exception of their σ1 proteins, encoding T1 and T3 σ1 proteins, respectively.19 While T1SA+ and T3SA+ can engage SA, T1SA- and T3SA- display diminished SA binding capacity by virtue of point mutations in the respective SA binding sites.19 T1 and T3 differ in their glycan affinities: T1 preferentially binds GM2, while T3 binds GM3. We first probed the interaction on model surfaces to establish the foundation for subsequent studies using living breast cancer cells. GM2 and GM3 model surfaces were prepared to mimic cell-surface glycans, and purified T1 or T3 virions were covalently attached at the AFM tip apex using a long polyethylene glycol (PEG) spacer (Figure 1c).9 Force−distance (FD) curves were recorded to assess the binding strength between virions and the glycan-terminated surface (Figure 3.

Figure 3. Binding and infectivity T1 and T3 reovirus. (a) Schematic representation of reovirus entry. The initial attachment of reovirus to cells involves specific binding of the viral σ1 protein to cell-surface glycans. (b) Fluorescent images of Alexa Fluor 488 virions bound to the cell surface and schematic representation of binding Western assays showing fluorescent virions bound to cells. The fluorescence intensity of (c) T1SA+ and (d) T3SA+ virions monitored on breast epithelial cells. In contrast to T1SA+ virus, T3SA+ binding increases on malignant cells. The fluorescence intensity of infected cells by (e) T1SA+ and (f) T3SA+ virions. Infected cells were detected using reovirus-specific rabbit polyclonal antisera and fluorescent secondary antibody. Infectivity of malignant cells by T3SA+ virions is significantly greater than that of nonmalignant cells. In contrast, infection by T1SA+ is cell line-independent. The error bars indicate SD with n = 6−17 for each condition. Distributions in panels c−f were evaluated using one-way ANOVA followed by posthoc Tukey’s HSD tests. *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.001, and n.s. nonsignificant.
Specific adhesion events were observed on 10–15% of retraction FD curves at rupture distances >5 nm (corresponding to the extension of the PEG linker) for GM2-T1SA+ as well as GM3-T3SA+ interactions (Figure 1f). Cross-probing of the interactions (T1SA+ binding to GM3 and T3SA+ binding to GM2) did not yield significant binding events (<5%). Using AFM tips functionalized with viruses incapable of SA-binding (T1SA-, T3SA-) on GM2 or GM3 model surfaces, respectively, also did not yield binding events. These results confirm that reoviruses engage sialylated glycans in a serotype-specific manner, as previously shown using glycan array screening.20 To further study GM2–T1SA+ and GM3–T3SA+ interactions from a kinetic point of view, binding events were analyzed at various retraction speeds of the AFM tip, enabling exploration of the binding force over a wide range of loading rates (LRs). For each individual curve (Figure 1d), the binding force, the magnitude of the force just before the bond ruptures, and the LR, the slope of the curve before the rupture on the force versus time curve, are extracted and displayed in a dynamic force spectroscopy (DFS) plot. Using the physiologically relevant direction-of-force application, the GM2-T1SA+ complex withstood forces in the range of 25–400 pN (Figure 1g), consistent with values previously reported for the GM3-T3SA+ interaction (Figure 1h). According to the Bell–Evans (BE) model,21 the GM2–T1SA+ interaction can be described as a simple two-state model in which the bound state is separated from the unbound state by a single energy barrier located at distance \( x = 0.56 \pm 0.04 \) nm and crossed at a transition rate of \( k_{\text{off}} = 0.61 \pm 0.21 \) s\(^{-1}\), significantly higher than the value measured for the GM3–T3SA+ interaction (Figure 1h). We also observed bivalent and trivalent interactions in both cases, which appeared as uncorrelated bonds loaded in parallel, as confirmed by the predictive Williams–Evans (WE) model (Figure 1g,h dashed curves II and III). These multivalent interactions are most likely established between \( \alpha \) molecules on a single T1SA+/T3SA+ virion attached to the AFM tip and multiple SA molecules immobilized on the surface. This hypothesis is supported by the following observations: (i) \( \sigma \) is a trimer with three binding sites; (ii) each virion has multiple copies (up to 12, corresponding to the virion icosahedral 5-fold vertices) of the \( \sigma \) trimer; (iii) the tip apex contains only one or two virions; and (iv) the unbinding occurs in a single step (a single rupture peak observed in the FD curves). Collectively, these in vitro experiments confirm that T1SA+ and T3SA+ virions specifically interact with serotype-specific \( \alpha \)-SA glycans. Moreover, virions rapidly (in the ms range) establish multivalent bonds with \( \alpha \)-SA glycans, providing the virion its first stable anchorage to the cell surface.

**T3SA+ Reovirus Preferentially Binds to Malignant Cells.** Cancer onset is accompanied by changes in the mechanical phenotype of cells that soften23,24 and increase their plasticity, allowing invasion of surrounding healthy tissue.25 This property can be used as a biomarker to diagnose tumor formation.26 To validate MCF-10A, MCF-7, and MDA-MB-231 cell lines as a model of breast cancer progression, we probed their mechanical properties using AFM (Figure 2a). Extraction of data from individual FD curves obtained in AFM studies (Figure 2b) allows simultaneous recording of height (Figure 2c), Young’s modulus (Figure 2d), and adhesion maps (Figure 2e) under physiologically relevant conditions as well as their display in the form of maps formed by color-coded pixels. Height maps revealed that cell size varies between 30 and 40 \( \mu \)m with a maximum height of \( \sim 6 \mu \)m for MCF-10A and MCF-7, and \( \sim 10 \mu \)m for MDA-MB-231 cells. These parameters are consistent with the observation that rounder cell shapes are associated with metastatic potential.27 The analysis of the Young’s modulus indicated 2-fold significant differences between cell lines, thus correlating with the degree of malignancy (Figure 2f).

After validation of cell malignancy based on phenotype and mechanics, we tested whether binding affinities of T1SA+ and T3SA+ were dependent on cell malignancy, perhaps due to altered glycan expression during cancer progression.15,32,28 To probe virus binding to cell surfaces, AFM tips were functionalized with reovirus T1SA+ or T3SA+ virions as described.29 Specific adhesion events between the AFM tip functionalized with T3SA+ virions and the cell surface are displayed as bright pixels on the adhesion maps. To confirm the specificity of these interactions, we conducted independent control experiments using AFM tips functionalized with T1SA- or T3SA-, which do not engage \( \alpha \)-SA. We analyzed the adhesion maps recorded using the three breast epithelial cell lines and quantified the binding probability as the percentage area exhibiting specific reovirus binding events (Figure 2g). T1SA- and T3SA- did not display significant binding to cells, indicating that SA engagement is required for reovirus attachment to the cell lines under investigation. For T3SA+ virions, the binding probability significantly increased on malignant cells. Binding frequency was 3–5-fold higher on malignant cells with 4.9 ± 3.3%, 20.8 ± 9.1%, and 14.2 ± 7.4% for MCF-10A, MCF-7, MDA-MB-231, respectively. Surprisingly, the binding probability for T1SA+ serotype was not influenced by the cell malignancy (Figure S1), suggesting that binding of T3SA+ but not T1SA+ to the cell surface depends on cell malignancy.

**Reovirus Infectivity of Breast Cancer Cells Correlates with Attachment.** As we observed a higher binding probability of T3SA+ reovirus virions for malignant cells, we sought to determine whether the higher binding probability results in more efficient infectivity. We incubated cells with T1SA+/T1SA- and T3SA+/T3SA- reovirus virions for different intervals and quantified virus attachment to the cell surface and productive infection (Figure 3a). Virion binding was quantified after a 45 min incubation at 4°C by assessing the fluorescent intensity of virions attached to the three cell lines (Figure 3b). In accordance with binding frequency results using AFM, we observed clear differences between T1SA+ and T3SA+ virions. While fluorescence intensity of T1SA+ reovirus virions did not differ significantly among the cell lines, strong differences were observed in the case of T3SA+ (Figure 3c,d). Malignant cells (mild and metastatic) show at least 2-fold higher binding than nonmalignant cells. These results confirm observations made using AFM and single virion particles and indicate that T3SA+ reovirus preferentially binds malignant cells.

After 24 h of incubation at 37°C, we quantified infectivity by each of the reovirus strains studied. The percentage of cells infected by T1SA+ virions was comparable regardless of cell malignancy (Figure 3e). In contrast, T3SA+ virions infected malignant cells more efficiently than the nonmalignant counterparts (Figure 3f). In concordance with previous results, T3SA- and T1SA- displayed a lower level of binding to and infection of all three cell types. To further test whether SA glycans contribute to T3 reovirus binding and infectivity, we conducted binding and infectivity experiments following treatment of cells with neuraminidase (NA, Figure S2a), which specifically cleaves SA expressed on the cell surface. NA treatment was associated with a significant decrease in virus attachment (up to 65%, Figure 3f).
and infectivity (up to 43%, Figure S2b), confirming an essential function for cell-surface SA in reovirus binding and infection of cancer cells. Together, these results confirm that T3SA+ virions preferentially bind and infect more malignant cells in a SA-dependent manner.

**Malignant Cells Express Higher Levels of the α-SA Glycans Bound by T3 Reovirus.** To determine whether the difference in infectivity of breast cancer cells by T1SA+ and T3SA+ is attributable to the glycan profile expressed by malignant cells, we conducted staining and immunoblot experiments to quantify glycans that specifically interact with T1SA+ and T3SA+ reovirus virions. We used *Helix pomatia* agglutin (HPA) lectin, which binds GalNAc, recognized by T1SA+ reovirus,30,31 *Sambucus nigra* agglutinin (SNA) lectin, which binds α2,6-SA residues, recognized by T3SA+ reovirus,30,32 and *Maackia amurensis* lectin (MAL) lectin, which binds α2,3-SA, a ligand for both T1SA+ and T3SA+ reoviruses.30,33 The distribution of HPA lectins (GalNAc) was similar on the three cell types (Figure 4a). Interestingly, SNA (α2,6-SA) and MAL (α2,3-SA) signals distributed to cellular junctions for nonmalignant MCF-10A cells and on the basal and apical surfaces for mildly malignant MCF-7 and metastatic malignant MDA-MB-231 cells (Figure 4a). We also compared expression of GalNAc and α-SA by the three cell types using immunoblotting. GalNAc expression by the three cell types tested was comparable (Figure 4b). However, α-SA expression by the malignant cells (MCF-7 and MDA-MB-231) was greater than that by the nonmalignant cells (MCF-10A) (Figure 4c,d). We extracted fluorescence intensity of the lectin staining (Figure 4e) to correlate cell-surface glycan expression to infectivity. T3SA+ infectivity increased with the expression of α2,3-SA (MAL) and α2,6-SA (SNA), which correlated well with cell malignancy (Figure 4f). In contrast, T3SA+ infectivity did not correlate with GalNAc expression. Thus, α-SA overexpression on malignant cells is associated with T3 reovirus infectivity.

**Addition of α-Sialylated Glycans Enhances Reovirus Infectivity.** Although SA engagement may provide initial attachment of reovirus to the cell surface, the virus must bind a specific receptor such as JAM-A to trigger cell entry.30 Since incubation of virions with α-sialylated glycans triggers a
conformational change in σ₁ that enhances virion binding to JAM-A,9 we determined whether cancer cell infectivity by reovirus also could be enhanced by glycan addition. To test this hypothesis, we quantified reovirus attachment to the cell surface and subsequent infectivity in the presence of α-sialylated glycans in the cell culture medium. We incubated cells with either SA (Neu5Ac), a short α-sialylated glycan (LSTa), or a similar glycan lacking α-SA (LNnT). The addition of Neu5Ac and LSTa in the medium increased both reovirus attachment (Figure 5a−c) and infectivity (Figure 5d−f). However, addition of LNnT had no effect on reovirus binding and infectivity, confirming that α-SA glycans specifically promote reovirus binding and cell entry. Collectively, these results show that viral infection efficiency can be enhanced by the addition of soluble α-SA glycans.

Finally, we determined whether reovirus induces apoptosis in cancerous cells. T3SA+ virions were adsorbed to cells, and apoptosis was assessed by acridine orange staining 24 h post infection (Figure S3). T3SA+ infection of breast cancer cells induced chromatin condensation and morphological changes associated with apoptosis in approximately 21% of MCF-10A cells (30% with SA addition, 74% of MCF-7 cells (83% with SA addition) and 57% of MDA-MB-231 cells (67% with SA addition) 24 h postinfection. These results indicate that reoviruses efficiently infect cancerous breast cells and promote cell death by apoptosis. Moreover, addition of free α-SA glycans enhances cell apoptosis, which provides an appealing approach to improve the use of reoviruses as oncolytic agents.

DISCUSSION

Breast cancer has surpassed lung cancer as the most commonly diagnosed cancer in women with an estimated 2.3 million new cases annually worldwide.34 TNBC accounts for approximately 15% of breast cancers and has a higher relapse rate and shorter average survival interval after metastasis than other breast cancer subtypes.35 Although targeted therapies have been successful against hormone receptor-positive and HER2-positive breast cancer, the absence of these receptors on TNBC cells has limited the options for cytotoxic chemotherapy, radiotherapy, and surgery.36 The severity of TNBC prompts an urgent need for targeted therapeutics against cancer of this type. We confirmed here that breast cancer cells express higher levels of α2,3-SA relative to less metastatic cell lines and suggested that this characteristic can be exploited in the development of new therapeutics. In this context, reovirus appears as a promising candidate due to its capacity to target and kill malignant cells.13−15,17 We evaluated how overexpression of glycans in cancerous cells influences reovirus oncolytic activity. Using AFM in combination with binding and infectivity, we compared two reovirus strains representing different serotypes, T1SA+ and T3SA+, and observed significant differences in their capacity to bind and infect three breast epithelial cell lines, MCF-10A (benign), MCF-7 (mildly malignant), and MDA-MB-231 (malignant metastatic, a TNBC cell line), which were chosen as a model of breast cancer progression. We demonstrated for the first time at the single-virion level that reovirus T3SA+, but not T1SA+, binding and infectivity
remarkably increased on malignant cells. We correlated these increased properties to changes in glycan distribution; while both SNA (α2,3-SA) and MAL (α2,6-SA) are mainly located between cellular junctions in nonmalignant MCF-10A cells, they are found more predominantly on the basal and apical surfaces for mildly malignant MCF-7 and metastatic malignant MDA-MB-231 cells. We also observed that GalNAc is expressed at comparable levels by the three cell types studied, explaining why reovirus T1SA+ does not bind preferentially to any of these cells. Thus, differences in the oncolytic capacity of T1 and T3 reoviruses appear to be attributable to the particular type of α-Sa glycans expressed on the malignant cells. Finally, we investigated whether the initial step of virus attachment to malignant cells and ensuing infectivity can be specifically enhanced by the addition of short α-sialylated glycans. Our results demonstrate that the addition of α-sialylated glycans, SA, and α-sialylated tetraose, facilitates virus attachment and cell entry. As virus binding and cell entry are the first steps in virus infection and likely to be limiting, the use of short sialylated glycans appears to offer an opportunity to improve reovirus-based oncolytic treatment. Our results provide a direct link between glycan expression, reovirus binding, and reovirus infectivity in the context of breast cancer, providing a new basis for the development of more effective oncolytic treatments.

ASSOCIATED CONTENT

Supporting Information
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Additional details on the methods and Figures S1–S3 (PDF)

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D.M. and D.A. conceived the project. D.M. and M.K. performed the experiments. D.M. and M.K. analyzed the data and wrote the main manuscript text and prepared figures. P.A. and D.M.S. engineered the viruses. D.M., M.K., A.C.D., P.A., D.M.S., T.S.D., and D.A. contributed to the interpretation of the results and improved the manuscript and figure presentations. All authors wrote the paper.

Notes
The authors declare no competing financial interest.

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