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Investigating the role of mucin in the delivery of nanoparticles to cellular models of human cancer disease: an *in vitro* study

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Abstract

Mucin, a glycosylated protein, is aberrantly overexpressed in a variety of tumor cells. The glycoprotein mesh decreases the rate of intracellular drug uptake and effectiveness. We investigated the influence of the mucin mesh on the cellular uptake of anti-MUC1 antibody and nanoparticles by fluorescence spectroscopy and microscopy. A glycosylation inhibitor (benzyl-α-GalNAc) was employed to regulate mucin glycosylation events. In our panel of pancreatic cell lines, only PANC-1 cells exhibited a significant increase in the uptake of liposomes following glycosylation inhibition, resulting in improved cytotoxicity of gemcitabine-loaded liposomes. Interestingly, areas devoid of liposome uptake were observed for pancreatic cancer cell lines PANC-1, Capan-1, and Capan-2; however, these restricted regions could be diminished for PANC1 cells only. In conclusion, investigating the reason(s) for differential cellular uptake of nanoparticles, in association with the production of mucin glycosylation mesh, should provide valuable leads to the future development of nanomedicine for cancer treatment.

Key words: Cancer; Mucin; Glycosylation; Liposomes; Microspheres; Drug delivery; Nanotechnology; Tumor barriers; Pancreatic cancer

Mucin provides a natural protective barrier to epithelial cells against a variety of potentially harmful environmental assaults.[1,2] The glycoprotein consists of oligosaccharides that are mainly O- or N- linked to a protein backbone.[3,4] The post-translational modification (i.e., glycosylation, sialylation, and sulfation) of the protein core frequently occurs in mature populations of mucin in a cell type-specific manner.[5–7] Type-O-glycosylation occurs in approximately 50-80% of cases, with N-glycosylation occurring approximately 20%-30% of the time.[8,9] Translational modification of the core protein depends on enzymes such as glycosyltransferase, sulfotransferases, and fucosyltransferase.[10,11]

In normal epithelial cells, the mucin glycoprotein is expressed mainly on the cell surface.[12] However, by the action of several insults affecting cellular integrity the well-defined expression of mucin is lost, resulting in an aberrant expression and the formation of a mucin mesh surrounding the cell.[13,14] During type-O-glycosylation, N-acetylgalactosamine (also known as GalNAc) is the first sugar moiety to attach the protein core followed by the remaining glycans.[15] Any interruption to this process will impair the glycosylation event.[16,17] The type O-glycosylation inhibitor benzyl-α-GalNAc (an analogue for N-acetylgalactosamine) is a beneficial agent in the study of glycoprotein expression and function, given its ability to safely regulate the production of mucin glycosylation in viable cells.[14,18,19]

An estimated 17 mucin genes have been identified and categorized as either membrane-bound or secreted.[20] The membrane-bound variety has a single transmembrane domain spanning the cellular membrane, and functions mainly as a protective layer due to the presence of the glycosylated tandem repeats.[21,22] Secreted mucins form a physical barrier in the form of a mucus gel, offering protection to the lungs and the gastrointestinal tract.[23] Among the various mucin genes, MUC1 (a membrane-bound protein) is one of the most widely investigated isoforms.

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Table 1
Non-toxic concentrations for mucin glycosylation inhibitor (benzyl-α-GalNAc).

No.	Cell line	Organ tissue	Non-toxic concentrations of Benzyl-α-GalNAc employed (mg/ml)
1	Capan-1	Pancreas	0.4
2	Capan-2	Pancreas	1.0
3	HPAF-II	Pancreas	1.0
4	PANC-1 PANC-1 GFP	Pancreas	0.8
5	hTERT-HPNE	Pancreas	0.4
6	CRL-1777	Pancreas	0.8
7	ZR-75-1	Breast	2.0
8	CRL-2089	Breast	1.0
9	ChaGo-k-1	Lung	1.0
10	Colo-205	Colorectal	0.5
11	PC-3	Prostate	0.5
12	RC-13	Kidney	0.01
13	CAL-62	Thyroid	1.0

Previous work has shown that the mucin mesh acts as a sieve; allowing the passage of molecules small enough (i.e., gas and nutrients) to pass through the spaces in between the mesh, while delaying the passage of relatively large molecules.[24–26] For example, the cytotoxic effect of 5-FU was significantly enhanced following inhibition of glycosylation *in vitro* and *in vivo*.[27,28] The silencing of MUC4 gene significantly improved the sensitivity of Capan-1 cells to bortezomib and gemcitabine.[29] To this date, it is not known whether conventional drug carrier molecules are affected by the mucin mesh. For this reason, we evaluated the baseline mucin expression level of a variety of tumor cell lines derived from different organ tissue environments. We screened the relative access of an MUC1 antibody to the cell surface following inhibition of O-glycosylation. Finally, we investigated whether the mucin mesh functions as a barrier impeding the cellular uptake of nanoparticles, using fluorescently labeled microsphere beads and conventional liposomes.

Methods

Benzyl-α-GalNAc was purchased from Sigma-Aldrich (St Louis, MO). Fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD227 (MUC1) monoclonal antibody was purchased from BD Pharmingen (San Jose, CA). Cell culture media EMEM, IMDM, RPMI-1640, DMEM, F-12 K, and McCoy's and trypsin-ethylenediaminetetraacetic acid were purchased from ATCC (Manassas, VA). Cell lines used were purchased from ATCC (Manassas, VA) and AntiCancer incorporation (San Diego, CA). Fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD227 (MUC1) monoclonal antibody was purchased from BD Pharmingen (San Jose, CA). Three different sizes (67 ± 2 nm, 116 ± 3 nm, and 990 ± 30 nm) of carboxylate modified fluorescently labeled microsphere beads (FluoSpheres®) were purchased from life technologies (Carlsbad, CA). The sizes of the microsphere beads were confirmed by using ZetaPals particle size and zeta potential analyzer (Brookhaven Instruments, New York).

Cell culture

Human and murine cancer and normal cell lines were used. Capan-1, Capan-2, PANC-1, PANC-1 GFP, and HPAF-II represent the pancreatic cancer lines. The hTERT-HPNE cell line represents the normal pancreatic duct (intermediary cells formed during acinar-to-ductal metaplasia). CRL-1777 represents normal pancreas/islet of Langerhans derived from a hamster. In addition to COLO 205 (colorectal tumor cells derived from a metastatic tumor site), ChaGo-K-1 (lung, bronchogenic carcinoma), RC13 (renal sarcoma-derived from metastatic site), PC-3 (prostate tumor cells derived from bone metastatic site), CAL-62 (thyroid, anaplastic carcinoma), and ZR-75-1 (ductal breast carcinoma cells derived from metastatic site-ascites) were maintained in their respective growth media. All cell lines were grown in a 5% CO_2 at 37 °C.

Preparation of Benzyl-α-GalNAc and drug solutions

The appropriate nontoxic concentrations of Benzyl-α-GalNAc for each cell line type were solubilized in suitable media prior to addition to cells seeded in well plates. To facilitate solubilization of Benzyl-α-GalNAc, vortexing was used in addition to incubation in a water bath at 37 °C.

Lipids and liposome preparation

Anionic liposomes were prepared using the thin film evaporation method. Briefly, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-[Phospho-rac-(1-glycerol)] DOPG, and cholesterol lipid stocks obtained from Avanti Polar Lipids (Alabaster, AL) were stored at −80 °C under an inert atmosphere. When preparing liposomes [DOPG/DOPC/chol/rhodamine- DPPE label (50:40:10)], the anionic charge was contributed by 50 mol% of the anionic lipid DOPG. The total lipid concentration was typically between 10 and 20 μmol/ml. The concentration of the fluorescent label rhodamine-DPPE was normally between 1-2 mol% of the total liposome preparation. The liposomes were prepared as previously reported.[30,31] Briefly, the respective mixtures were evaporated to dryness at 45 °C in a pyrex tube using a rotary evaporator to form a thin film. The film was then hydrated with 1 ml of 1X PBS to form multilamellar liposomes. Liposomes were vortexed intermittently and put in a water bath set at 45 °C and in ice for 30–60 min increments, then incubated in a water bath overnight at 37 °C. To obtain the desired sizes, the liposomes were next sonicated using a bath-type sonicator (Laboratory Supplies Corporation, Hicksville, NY, USA). Next, the liposomes were extruded using 2 filters with a 50 nm or 100 nm pore size. The larger size liposome was produced spontaneously without sonication or extrusion. Lastly, the particle sizes of liposomes were measured using ZetaPals particle size and zeta potential analyzer (Brookhaven Instruments, New York). The size ranges of the prepared liposomes were 60-80 nm, 100-120 nm, and 450-650 nm.