A Novel Therapeutic Strategy for Pancreatic Cancer: Targeting Cell Surface Glycan Using rBC2LC-N Lectin–Drug Conjugate (LDC)

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Abstract

Various cancers, including pancreatic ductal adenocarcinoma (PDAC), remain intractable even with costly tumor-targeting antibody drugs. Because the outermost coatings of cancer cells are composed of cell-specific glycan layers (glycocalyx), lectins, proteins with glycan-binding potential, were evaluated for possible use as drug carriers in PDAC treatment. A human PDAC cell line with well-to-moderately differentiated properties (Capan-1) was subjected to lectin microarray analysis to identify specific lectin–glycan pairs. The selected lectin was fused with a bacterial exotoxin for the construction of a lectin–drug conjugate (LDC), and its safety and antitumor effects were evaluated. A specific affinity between a recombinant bacterial C-type lectin (rBC2LC-N) and Capan-1 was identified, and its positivity was confirmed in 69 human samples. In contrast to the belief that all lectins mediate harmful hemagglutination, rBC2LC-N did not cause hemagglutination with human erythrocytes and was safely administered to mice. The 50% inhibitory concentration of LDC to Capan-1 (1.04 pg/mL = 0.0195 pmol/L) was 1/1,000 lower than that reported for conventional immunotoxins. The intraperitoneal administration of LDC reduced the tumor weight from 390 to 130.8 mg (P < 0.01) in an orthotopic model and reduced the number of nodules from 48 to 3 (P < 0.001) and improved survival from 62 to 105 days in a peritoneal dissemination model (P < 0.0001). In addition, the effect of LDC was reproduced in nodules from patient-derived PDAC xenografts through intravenous injection. Herein, we show the concept of utilizing lectins as drug carriers to target glycans on the cancer cell surface, highlighting new insights into cancer treatments. Mol Cancer Ther; 17(1); 183–95. ©2017 AACR.

Introduction

Cancer-targeting antibody drugs, including antibody-drug conjugates (ADC; refs. 1, 2), are attractive therapies, but their clinical success rates have been limited (3). The targets of these drugs are usually peptides of cell transmembrane proteins; however, it is important to note that cell surfaces are covered by a glycan layer that is referred to as the glycocalyx (4). The glycomes of cancer cell surfaces are often unique, with aberrant glycosylation, including sialylation, fucosylation, and its safety and antitumor effects were evaluated. A specific affinity between a recombinant bacterial C-type lectin (rBC2LC-N) and Capan-1 was identified, and its positivity was confirmed in 69 human samples. In contrast to the belief that all lectins mediate harmful hemagglutination, rBC2LC-N did not cause hemagglutination with human erythrocytes and was safely administered to mice. The 50% inhibitory concentration of LDC to Capan-1 (1.04 pg/mL = 0.0195 pmol/L) was 1/1,000 lower than that reported for conventional immunotoxins. The intraperitoneal administration of LDC reduced the tumor weight from 390 to 130.8 mg (P < 0.01) in an orthotopic model and reduced the number of nodules from 48 to 3 (P < 0.001) and improved survival from 62 to 105 days in a peritoneal dissemination model (P < 0.0001). In addition, the effect of LDC was reproduced in nodules from patient-derived PDAC xenografts through intravenous injection. Herein, we show the concept of utilizing lectins as drug carriers to target glycans on the cancer cell surface, highlighting new insights into cancer treatments. Mol Cancer Ther; 17(1); 183–95. ©2017 AACR.

Materials and Methods

Cell lines

A total of six human pancreatic cancer cell lines with various cell differentiation states in their origin were used. AsPC-1 (CRL-1682,
differentiated for Capan-1; and those of the other morphologies of their clinical PDAC origin were as follows: well differentiated for Capan-1; and those of the other five lines were poorly or poorly moderately differentiated (13). Capan-1, MiaPaCa-2, and AsPC-1 cells were verified by STR-PCR in March 2016 (report number: KBN0366, National Institute of Biomedical Innovation). The remaining cell lines were used within 6 months of purchase and were tested for contamination with mycoplasma.

**Patient samples and tissue collection**

Fresh human pancreatic cancer tissue was obtained with patient consent as approved by the Research Ethics Board of the University of Tsukuba. A total of 69 resected pancreatic adenocarcinoma specimens (7 poorly differentiated (P/D) cases, 53 moderately differentiated (M/D) cases, and 9 well-differentiated cases) were used in this study. For the use of these clinical samples for research purposes, written informed consent was obtained from all patients, and approval was obtained from the Tsukuba Clinical Research & Development Organization (T-CReDO protocol number: H28-90).

**Lectin microarray**

The high-density lectin microarray was prepared as previously described (14). Briefly, proteins from each cell line were prepared from whole-cell lysates of six different wells and fluorescently labeled with the monoreactive dye Cy3 (GE Healthcare). Then, 0.5 µg/mL Cy3-labelled lysate was added to each well of a microarray plate, and the plate was incubated at 20°C overnight. Fluorescence images were then acquired using an evanescent field-activated fluorescence scanner (GlycoStation Reader; GlycoTechnology Ltd.). The fluorescence signal of each spot was quantified using Array-Pro Analyzer version 4.5 (Media Cybernetics), mean-normalized, log-transformed, and analyzed via the average linkage method using Cluster 3.0 (yellow: high; black: intermediate; blue: low).

**Lectin staining**

**Lectin histochemistry.** Antigen retrieval in 2-µm slide sections of formalin-fixed and paraffin-embedded (FFPE) tissues was performed by autoclaving, endogenous peroxidase activity was blocked with 3% H2O2 with methanol, and horseradish peroxidase-labeled rBC2 was applied and visualized by applying the chromogen diaminobenzidine. The clinical cases were judged as 0, 1+, 2+, 3+ with regard to rBC2 reactivity, and the correlation with cell differentiation was assessed.

**Live-cell staining by rBC2 lectin.** Live cells were incubated for 12 hours in medium containing FITC-conjugated rBC2LC-N (1 µg/mL); images were captured using a BIOREVO BZ-X710 fluorescence microscope (KEYENCE).

**Production of rBC2-PE38**

The recombinant N-terminal domain of BC2L-C (rBC2LC-N; 156 amino acids) was fused to a truncated form of the catalytic domain of pseudomonas exotoxin (PE) A (399–613 residues; 215 amino acids) carrying a C-terminal 6×His tag (HHHHHHH) and a KDEL sequence via a ten-amino-acid linker (GSG3)2 (Fig. 2A). The generated rBC2-PE38 (371 amino acids) was expressed in E. coli and purified via one-step affinity chromatography on an Ig-fucose-Sepharose column. The yield was 10 mg/L of bacterial culture. rBC2-PE38 was detected as a major band at the predicted molecular weight of 54 kDa by SDS-PAGE in the presence and absence of 2-mercaptoethanol (Supplementary Fig. S1).

**Cell assays**

**Lectin binding analysis.** Cells were harvested and stained with various concentrations of rBC2-FITC for 30 minutes on ice. The MFI of the cells was determined using an LSR Fortessa X-20 flow cytometer. The data were analyzed using a nonlinear regression fitting program in GraphPad Prism 6 (GraphPad Software Inc.).

**Hemagglutination assay**

Blood samples were obtained from four healthy volunteers of each blood type, i.e., A, B, O, and AB. A 5-ml aliquot of freshly whole blood was washed 3 times with 20 to 25 mL of PBS (centrifugation at 500 × g for 5 minutes, followed by removal of the plasma and white cell ghost layer at the top of the pellet). The suspension of untreated (without using trypsin) erythrocytes was then mixed with an equal volume of incubation buffer (0.1 mol/L acetate buffer containing 1 mmol/L CaCl2, pH 5.5) containing 1 unit/mL neuraminidase (from C. welchii Sigma N2876) and incubated at 37°C for 1 hour with occasional shaking. All samples were again washed 3 times with PBS and then prepared as a 2% (v/v) erythrocyte suspension in PBS. Concanavalin A or rBC2 lectin solution (50 µg/mL) was applied to the left well of a U-shaped 96-well plate, and a series of 2-fold dilutions was prepared in a horizontal line. The same volume of erythrocyte suspension was added to each well, and agglutination was observed.

**Toxicology study of rBC2-PE38 (LDC) in wild-type mice**

To evaluate the LD50 (50% lethal dose) of LDC, female wild-type mice (5–6 weeks old, 18–20 g, Charles River Laboratories International, Japan) were given an intraperitoneal (i.p.) or intravenous (i.v.) injection with a single dose of 1.0 to 15.0 µg of rBC2/mouse or rBC2-PE38/mouse (50 to 750 µg/kg) in 300 µL of PBS. The mice were observed for survival until day 14.

**Animal models of PDAC**

Animal experiments were conducted in compliance with the ethical regulations approved by the Animal Care Committee,
University Health Network, University of Tsukuba, Tsukuba, Ibaraki, Japan.

Subcutaneous cell xenograft model. A total of 3 × 10⁶ Capan-1 or SUIT-2 cells were subcutaneously injected into female nude mice (BALB/c nu/nu, 6–8 weeks old, CLEA Japan).

Subcutaneous PDX model. Fresh human pancreatic cancer tissue originated from a nodule of liver metastasis of clinical PDAC that showed moderately-to-poorly differentiated morphology was implanted into subcutaneous pockets on the back skin of CB17/Icr-scid/SCID mice (female, 6 to 8 weeks old, CLEA Japan). After the establishment of first-generation subcutaneous nodules, the tumor nodules were minced into 2-mm cubic fragments, and 3 pieces were implanted into subcutaneous pockets on the back skin of SCID mice. These PDX nodules show moderately differentiation in morphology.

Pancreatic orthotopic model. Nude mice were anesthetized with isoflurane, a laparotomy was created using a left lateral abdominal incision, and the pancreas was exteriorized. Four tumor pieces (approximately 2 mm in diameter) obtained from peritoneal disseminated Capan-1 nodules were transplanted to the body of the pancreas and secured with absorbable surgical sutures.

Peritoneal dissemination model. Nude mice were inoculated with Capan-1 or SUIT-2 cells by i.p. injection of 2.0 × 10⁶ cells suspended in 100 μL of PBS. On day 14, 2 mice were sacrificed to confirm the establishment of disseminated cancer nodules. The remaining mice were then randomly allocated to four groups.

rBC2-PE38 (LDC) treatment for in vivo PDAC mouse models Direct injection (i.d.) to subcutaneous cell xenograft models and a PDX model. A d.i. of 40 ng, 1 μg, or 5 μg of LDC in 100 μL of PBS was performed a total of 4 times near the subcutaneous tumors on days 14, 18, 22, and 26. For the control, 100 μL of normal saline was injected. The tumor volume was determined every day for 2 weeks using the following formula: (width)² × (length)/2. On day 34, the subcutaneous tumors were excised for weight measurements.

i.p. and i.v. injection of LDC to subcutaneous PDX models. A total of 1 μg LDC was administered in 100 μL of PBS via i.p. injection (n = 4 each) or i.v. injection via the tail vein for a total of 8 times on days 14, 18, 22, 26, 29, and 31. On day 36, the subcutaneous nodules were excised for weight measurements.

i.p. and i.v. injection of LDC to orthotopic models. The mice were treated and evaluated on a treatment schedule identical to that of the cell xenograft models, using 1 or 5 μg of LDC (n = 5 each). On days 21, 24, 28, and 31, 1 μg of LDC was administered i.p. or i.v. via the tail vein. On day 45, the mice were sacrificed for the observation of abdominal status and tumor weight measurement.

i.p. and i.v. injection to peritoneal dissemination models. The Capan-1 peritoneal dissemination mouse models were treated by injection of 1 μg of rBC2 lectin without PE38, 40 ng of LDC, or 1 μg of LDC in 100 μL of PBS by i.p. or i.v. injection via the tail vein a total of 4 times on days 14, 18, 22, and 26 (n = 4 each). On day 30, the mice were sacrificed for manual counting of the disseminated nodules. The Kaplan–Meier survival curves were calculated using 36 nude mice. At 14 days after the Capan-1 cell injection, 4 groups of mice (n = 9 each) were treated on days 1, 5, 9, and 13 with control, 40 ng of LDC via i.p. injection, 1 μg of LDC via i.p. injection, or 1 μg of LDC via i.v. injection.

Statistical analyses

A heat map of the clustering analysis results was constructed using Java TreeView. Differences in the lectin signal between the two arbitrary data sets were evaluated using Student t test in SPSS Statistics 21.0 (SPSS Inc.). Significantly different lectin signals or glycosyltransferase expression levels were selected if they satisfied a familywise error rate (FWER) of <0.001 according to the Bonferroni method. Applied statistics included the unpaired Student t test and the Mann–Whitney U test. P < 0.05 was considered to indicate significance. Kaplan–Meier curves (statistically analyzed by the log-rank test) and LDC IC50 values were calculated using GraphPad Prism 6; other analyses were performed using SPSS 21.0 (SPSS Inc.).

Results

Selection of PDAC cell lines that display clinical PDAC and cancer stem-cell-like characteristics

For cancer cell glycome analysis, clinical PDAC samples are limited in terms of sample availability and quality due to the typical histological characteristics of PDAC, which include the presence of epithelial cancer cells forming ductal glands (g+) and abundant stromal components (s+; Fig. 1A). We therefore began the experiments by selecting a cell line that represents the dominant morphological properties of clinical PDAC when grown in vivo. Only Capan-1 cells strongly exhibited organized gland formation (g++) and dense stromal proliferation (s++; Fig. 1B). The expression of the known pancreatic cancer stem cell markers CD24, CD44, EpCAM, and CD133 (15, 16), as revealed by flow cytometry analysis, indicated that the Capan-1 line was positive for 3 markers and was the only cell line to show CD133 positivity (Supplementary Fig. S2). Therefore, we considered Capan-1 to be a rare PDAC line that maintains well-to-moderately differentiated tumor features and thus represents the dominant morphology of clinical PDAC characteristics. In fact, although most clinical human PDACs are well or well-to-moderately differentiated cancers according to histology, and poorly or moderately differentiated cancers represent only 10% to 15% of cases, most available PDAC cell lines are derived from poorly or moderately differentiated cancers (13).

High-density lectin microarray analysis of six types of PDAC cell lines

Glycan expression in Capan-1 cells, in which we have focused as the tester cell line that may represent PDAC glycan expressions, was compared with that in five other cell lines using high-density lectin microarrays (14). A representative heat map demonstrated that Capan-1 and BxPC-3 cells cluster on the same branch of a tree diagram, separate from the branch that includes the other four cell lines (Fig. 1C). Of the 96 lectins tested, the top 10 lectins with significantly different signal intensity in the Capan-1 cell line were identified; these included 8 lectins with increased reactivity and 2 lectins with decreased reactivity (Supplementary Fig. S3 and Table S1). The most prominent difference was found in the case of the rBC2LC-N (recombinant N-terminal domain of BC2L-C) lectin
(Fig. 1D), a TNF-like lectin that was originally identified in the gram-negative bacterium Burkholderia cenocepacia and that specifically binds to fucosylated glycan epitopes of H type 1/3/4 trisaccharides (17).

The rBC2 LC-N lectin exhibited specific affinity for Capan-1 cells and clinical PDAC samples

The affinity between the rBC2LC-N lectin and Capan-1 cell glycans was further confirmed by live-cell staining (Fig. 1E), flow cytometry analysis using FITC-labeled rBC2LC-N (Fig. 1F; Supplementary Fig. S4), and histochemical staining of an in vivo mouse cell-derived xenograft model with labeled rBC2LC-N (Fig. 1G; Supplementary Fig. S5). Each experiment revealed similar strong reactivity of the rBC2LC-N lectin to Capan-1 cells (Fig. 1E–G). To address whether reactivity with the rBC2LC-N lectin is specifically associated with Capan-1 cells or whether it can be expanded to general clinical PDAC, we analyzed 69 human clinical PDAC specimens by rBC2LC-N lectin histochemical staining. The representative staining patterns of three clinical PDAC of poorly (P/D), moderately (M/D) and well-differentiated (W/D) cases are shown in Fig. 1H. In 7 P/D cases, 6 were weakly positive (+) for rBC2 staining, whereas in 9 W/D cases, 7 were strongly (+++) or very strongly (3+) positive. M/D cases comprised the majority (53 cases) of the 69 clinical PDAC cases, and their rBC2 staining was as follows: 1+ in 18 cases, 2+ in 25 cases, and 3+ in 10 cases. The surrounding stromal components were negative for rBC2 staining (Fig. 1H).

For the top 10 lectins with the highest differences in specificity, lectin histochemical analysis of serial sections of clinical PDAC specimens demonstrated that rBC2LC-N showed the most prominent contrast in terms of its intensive affinity for cancer cells and its lack of affinity for stromal fibroblast cells (Supplementary Fig. S6). Our data indicate that a fucosylated glycan epitope (H type 1/3/4) that is recognized by the rBC2LC-N lectin may serve as a promising therapeutic target for PDAC.

Construction of LDC and its cytotoxic activity in vitro

With the aim of utilizing the rBC2LC-N lectin (rBC2) as a therapeutic drug carrier, a 38-kDa region of the catalytic domain of Pseudomonas aeruginosa exotoxin A (PE A) was fused with the lectin sequence to construct LDC (rBC2-PE38; ref. 18; Fig. 2A). The dissociation constant (Kd) values for rBC2 indicated high-affinity binding of the conjugate only to Capan-1 cells (Kd: 171.4 nmol/L, Fig. 2B). The cytotoxic effect of LDC was measured using an MTT assay; IC50 of LDC against Capan-1 cells was 1.04 pg/mL (0.0195 pmol/L, Fig. 2C), nearly 1,000 times lower than that of conventional immunotoxins, which generally have IC50 values on the order of ng/mL (Supplementary Table S2). The other five cell lines that displayed no affinity for the rBC2LC-N lectin showed moderate to negative cytotoxic effects (Fig. 2D). Because internalization into the cell cytoplasm is a key indispensable step for the cytotoxicity of PE toxin (19), the endocytosis of rBC2 lectins was observed. One hour after application of the conjugate, the cell surfaces of the Capan-1 cells were surrounded by rBC2 lectins (Fig. 2E). Endocytic vesicles containing rBC2 lectin were visible inside Capan-1 cells at 24 hours and more conspicuous at 48 hours. In contrast to Capan-1 cells that possess positive affinity for rBC2 lectins, the negative control cell line (SUIT-2), which has no affinity for rBC2 lectins, showed no fluorescence intensity on the cell membrane at 24 hours and no endocytic vesicles inside the cytoplasm at 48 hours.

Safety of intravitral administration of LDC (rBC2-PE38)

We questioned whether our selected lectin would be a safe and effective drug carrier that could be administered to cancer patients because lectins are often defined by their cell agglutination activities. We evaluated the reactivity of the rBC2LC-N lectin on the surface of noncancerous abdominal organs in mice (Fig. 3A) and confirmed that rBC2LC-N exhibited negative reactivity for the entire parietal peritoneum and the serosal membranes of visceral organs. The disseminated cancer nodules of Capan-1 cells were confirmed to be strongly positive for rBC2LC-N reactivity. Importantly, an in vivo hemagglutination test of erythrocytes from mice and a human volunteer (blood type A) demonstrated that the rBC2LC-N lectin did not induce hemagglutination even at a high concentration of 50 μg/mL, in contrast to the obvious aggregation caused by the concanavalin A (Con A) lectin at 1.56 μg/mL in mouse and human erythrocytes (Fig. 3B). LDC (rBC2-PE38) slightly affected hemagglutination at very high concentrations, as demonstrated by a positive result at 25 μg/mL in mouse erythrocytes and 12.5 μg/mL in human erythrocytes (Fig. 3B). Identical results were obtained for human erythrocytes of other blood types, including type B, AB, and O (Supplementary Fig. S7). In addition, the rBC2LC-N lectin staining of human red blood cells contaminating clinical PDAC slides revealed that all red blood cells were negative, indicating that human red blood cells are likely to be negative for the H type 1/3/4 motif (Fig. 3C; Supplementary Fig. S8). Notably, white blood cells (*red and blue nuclei) were also negative for rBC2LC-N reactivity. When the LDC 50% lethal dose (LD50) was analyzed by i.p. and i.v.

Figure 1. Screening for lectins that specifically bind to PDAC. A, The pathology of a typical PDAC specimen consists of a cluster of ductal spreading cancer cells with abundant stromal components, defined as gland formation (g+ +) and stromal induction (s+ +). B, The morphologies of the 6 pancreatic cancer cell lines in mouse xenografts with respect to gland formation (g+ +), + or −) and stromal induction (s+ +, or −). Capan-1 cells exhibited strong, organized gland formation (g+ +) and dense stromal proliferation (s+ +) and strong glandular induction (s+ +, or −). Capan-1 cells exhibited strong, organized gland formation (g+ +) and dense stromal proliferation (s+ +), Capan-1 cells exhibited strong, organized gland formation (g+ +) and dense stromal proliferation (s+ +), and Capan-1 cells exhibited strong, organized gland formation (g+ +) and dense stromal proliferation (s+ +). D, High-density lectin microarray analysis of 96 various lectins was performed on 6 PDAC cell lines (n = 6 protein samples for each cell line; technical replicates). A heat map of the clustering analysis (yellow: high; black: intermediate; blue: low) placed BxPC-3 and Capan-1 cells on an identical branch of the tree diagram (above the line). The position of the rBC2LC-N lectin is indicated by an arrow. D, For each of the 96 lectins assessed, the signal intensity in the Capan-1 cell line was compared with that of the other 5 cell lines. The rBC2LC-N lectin exhibited the most robust difference (two-tailed unpaired Student t test). The top 10 lectins that showed specific affinity differences in Capan-1 cells are described in Supplementary Fig. S3 and Table S1.

E, The specific activity of rBC2LC-N for Capan-1 cells in the lectin microarray was further confirmed by live-cell staining using FITC-labeled rBC2LC-N (scale bar, 50 μm). F, rBC2LC-N reactivity analysis by flow cytometry confirmed that Capan-1 cells displayed the highest affinity for rBC2LC-N, as indicated by an MFI of 6300. G, Lectin histochemistry of cell-based xenograft models demonstrated strong staining for rBC2LC-N in Capan-1 xenografts (brown signal and blue counterstaining with hematoxylin) and mosaic staining in BxPC-3 xenografts. The remaining 4 xenografts were negative for rBC2LC-N (scale bar, 100 μm). H, rBC2LC-N lectin histochemistry in human clinical PDAC specimens. Representative micrographs of 3 cases, including P/D with weak (+) staining, M/D with moderate (+) staining, and W/D with strong (++) staining, I, Among the 69 human clinical PDAC cases, P/D cases tended to show weak staining with rBC2, and W/D cases showed moderate to strong staining. The primary differentiation was M/D (53/69), which demonstrated similar distribution as W/D cases.
administration, acceptable values were estimated to be 7.14 µg/mouse (424.8 µg/kg) for i.p. and 7.22 µg/mouse (429.6 µg/kg) for i.v. (Fig. 3D). Administration of the BC2LC-N lectin without conjugated toxin did not cause death at any dose tested up to 15 µg/mouse (892.5 µg/kg), either i.p. or i.v. Based on these observations, we were confident that at least the i.p. injection, and likely also the i.v. injection, of LDC would be safe for mouse models in vivo.

Therapeutic effect of LDC in various mouse tumor models

Subcutaneous xenografts treated by local injection. In cell line-based models, Capan-1 xenografts (positive for the H type 1/3/4 glycan epitope) were significantly diminished by LDC d.i. (Fig. 4A1–3). However, regarding SUIT-2 xenografts, which exhibit no affinity to the rBC2LC-N lectin, LDC injection did not reduce the tumor sizes except when the dose reached 5 µg/mouse (Fig. 4B1–3). In a more clinically relevant PDX model, tumors decreased in size after LDC administration in a dose-dependent manner (Fig. 4C1–3).

Subcutaneous PDX xenografts treated by i.p. or i.v. injection. The antitumor effects of LDC administration via the i.p. or i.v. routes for subcutaneous tumors of the PDX model are shown in Fig. 4D1–3. Although these tumors grow rapidly in the control group (normal saline i.p.), LDC treatment groups that received a total of...
Figure 3. Safety of LDC administration. A, rBC2LC-N lectin reactivity to mouse organ surfaces that might be exposed to LDC administered via the i.p. route. Negative reactivity to the entire parietal peritoneum and the visceral peritoneum of hollow, solid and genitourinary organs (arrows) were confirmed, in contrast to the strong positivity in the Capan-1 disseminated cancer nodules. Positive reactivity to rBC2LC-N in epithelial cells on the internal surface of the hollow organs (strongly in the stomach, duodenum and fallopian tube and weakly in the small intestine and colon) can be ignored in i.p. administration because these cells do not encounter LDC when injected via the i.p. route. (scale bar, 100 μm) B, Hemagglutination assay of rBC2LC-N and LDC. The rBC2LC-N lectin alone did not induce erythrocyte aggregation from mice and a human volunteer, even at an extremely high concentration of 50 μg/mL. A positive control with concanavalin A (Con A) lectin demonstrated aggregation at 1.56 μg/mL. LDC (rBC2-PD38) slightly affected hemagglutination at the very high concentrations of 25 μg/mL in mouse and 12.5 μg/mL in human samples. Each test contained n = 3 technical replicates. Other blood types are shown in Supplementary Fig. S7. C, rBC2LC-N lectin staining of human red blood cells (Type A) contaminating clinical PDAC slides revealed that all red blood cells were negative, indicating that the rBC2LC-N lectin should not induce the aggregation of red blood cells. Notably, white blood cells (< with blue nucleus) were also negative for rBC2LC-N reactivity (scale bar, 100 μm). Other blood types are shown in Supplementary Fig. S8. D, The 50% lethal dose (LD50) of rBC2LC-N alone and LDC by i.p. (n = 10 wild-type mice for each concentration) and i.v. (n = 7) administration were evaluated. The LD50 of a single i.p. and i.v. administration of LDC was calculated to be 7.14 μg/mouse and 7.22 μg/mouse, respectively. By contrast, rBC2LC-N alone did not induce death at any dose tested up to 15 μg/mouse by both i.p. and i.v. administrations.
eight treatments showed reduced growth with either i.v. or i.p. administration. The average tumor weights at day 36 were 991 mg in the control group, whereas those of i.p. and i.v. administration groups were 540.6 and 686.6 mg, respectively (P < 0.01 and 0.05, respectively, relative to control).

**Pancreatic orthotopic xenografts treated by i.p. or i.v. injection.** The effects of 4 LDC treatments (1 μg/mouse) administered via i.p. or i.v. for orthotopic tumors are shown in Fig. 4E–I. The average tumor weights after i.p. and i.v. administration were 130.8 mg and 203 mg, respectively (P < 0.001 and 0.05, respectively, relative to the control value of 390 mg). Macroscopic metastases to the liver, spleen, or abdomen occurred frequently in the control groups (3–4/5 mice) but were rare in the LDC treatment groups (0–2/5 mice).

**Peritoneal dissemination models treated by i.p. or i.v. injection.** On day 14, peritoneal disseminated nodules had grown to a visible size and measured 1–3 mm (Fig. 5A). In the Capan-1 group, significantly fewer disseminated nodules were observed upon treatment with either 40 ng of LDC/mouse or 1 μg of LDC/mouse (Fig. 5B). In the SUIT-2 group, for which rBC2 exhibited no affinity, LDC had no effect (Fig. 5C). In the control groups, the peritoneal cavities of the mice contained abundant cancer cells, whereas there were no cancer cells in the peritoneal cavities of the mice in the 1 μg of LDC/mouse treatment group (Fig. 5D). At day 45, the weights of the mice were significantly improved both in i.v. and i.p. administrations (Fig. 5E). The effect of LDC administration occurred even after i.v. administration, as shown by the absence of disseminated nodules (Fig. 5F–I), ascites, and liver metastasis (Fig. 5F–I). Finally, we addressed whether glycans-targeting LDC therapy could cure PDAC in the mouse model of PDAC (Fig. 5G). The median survival of the control group was 62 days, with a 90-day survival rate of 0% (0/9). In contrast, administration of four i.p. injections of 1 μg of LDC resulted in a 90-day survival rate of 78% (7/9) and improved the median survival time significantly (105 days; P < 0.0001). The effects of four i.v. injections of 1 μg of LDC were comparable to those of i.p. injection, resulting in a 56% (5/9) 90-day survival rate and a median survival time of 90 days.

### Discussion

Thus far, the potential of lectins for in vivo cancer treatment has not been well tested, likely due to the common belief that all lectins mediate harmful hemagglutination. In this study, we found a specific affinity between the rBC2LC-N lectin and PDAC cell surface glycans (fucosylated glycan epitopes of H type 1/3/4 trisaccharide), and this lectin was shown to be a safe and efficient drug carrier in cancer treatment.

The identification of rBC2LC-N as the most highly reactive lectin in PDAC was an unexpected coincidence because we previously reported that rBC2LC-N specifically interacts with induced pluriotent stem (iPS) and embryonic stem (ES) cells but not with differentiated somatic cells (20). The serendipitous finding that an rBC2LC-N fucose-binding lectin displays affinity for PDAC and iPS/ES cells indicates that consensus glycan modification of membrane proteins or lipids containing H type 1/3/4 glycan on PDAC cells plays a crucial role in the formation and development of cancerous tissue. The specific affinity between LDC and cancer cell surface H type 1/3/4 glycan was confirmed by competitive inhibition by adding free rBC2LC-N lectin and free glycan (=fucose; Supplementary Fig. S9).

The critical question has been whether the rBC2LC-N lectin can safely be administered to cancer patients because lectins are initially defined by their blood agglutination activities (10). This concern was addressed using an *in vitro* hemagglutination assay; the results showed that the rBC2LC-N lectin did not induce the aggregation of human erythrocytes (Fig. 3B; Supplementary Fig. S7), and the negative rBC2LC-N staining of human red blood cells contaminating 69 clinical PDAC specimens is shown (Fig. 3C; Supplementary Fig. S8). This finding might be explained by the fact that the ABO blood type glycans on red blood cells contain predominantly H type 2 trisaccharide motifs in which Gal and GlcNAc are connected via beta-1-4 glycosidic bonds (21). rBC2LC-N is known to bind only type 1/3/4 trisaccharide motifs and not H type 2 motifs (17). The biosynthesis of H type 1 trisaccharide motif is completed via fucose addition to galactose in type 1 (Gal-GalNAc), or H type 3/4 are generated by fucosylation of type 3/4 disaccharide (Gal-GalNAc) by fucosyltransferases (Fig. 6A; ref. 17). Moreover, the rBC2LC-N lectin was administered to mice without notable adverse events, further indicating that this lectin alone does not induce blood coagulation *in vivo* (Fig. 3D). Based on our experimental data and the literature, we are confident that the rBC2LC-N lectin could be administered via the intravenous route without inducing hemagglutination.

The next issue should be the potential nonspecific attachment of rBC2LC-N to nontumorous normal tissues other than red blood cells. Fucose modification of glycans is one of the representative cancer-specific changes in glycosylation (5); however, fucosylated type 1/3/4 glycan epitopes are also found in nontumorous epithelial cells of the gastrointestinal, respiratory, and reproductive tracts (22). Our results obtained using rBC2LC-N lectin staining also revealed positive staining of epithelial cells in...
Figure 5.
Effectiveness of LDC for the treatment of PDAC peritoneal dissemination in a mouse model. A, At day 14 after i.p. injection of Capan-1 or SUIT-2 cells, many opal-colored nodules known as milky spots (arrows) were distributed on the peripheral margin of the small intestine (28). LDC was i.p. or i.v. injected 4 times. B, The mesenterium after treatment on day 30. In the Capan-1 model, numerous nodules (arrow) remained in the control and rBC2LC-N alone groups, whereas nodules were significantly and dose-dependently reduced in LDC-treated groups (n = 4). C, The number of nodules was not altered in the SUIT-2 model. (Continued on the following page.)
and O-linked glycans. The proximate GaNAc in O-linked glycan could be a source of the H Type III trisaccharide motif. The end glycolipid, i.e., GaNAc attached to the Globo H construct, could also be a source of the H type IV glycan motif. B, Schema of rBC2LC-N lectin binding on the cancer cell surface. Right, The previous failure of ADC strategies may be due to the one- to-one relationship between the cancer cell surface peptide antigen (blue star in brown glycoprotein) and the antibody, which results in the delivery of an insufficient amount of the drug to cancer cells. Left, In contrast, LDC binding sites could consist of multiple glycans on various glycoproteins and glycolipids, resulting in the delivery of more drug to the surface of target cells. In addition, the relatively large size of immunoglobulins tends to prevent effective internalization of antibody-based drugs; however, the relatively small (16-kDa) size of rBC2LC-N would facilitate effective endocytosis and the prominent cytotoxic effect of LDC.

Figure 6.

A, Schema of rBC2LC-N lectin binding sites. rBC2LC-N is a TNF-like lectin molecule identified from the gram-negative bacterium B. cenocepacia, and this molecule is revealed to be a fucose-binding lectin (17). Precise survey using a glycan array containing 377 glycans revealed that the promising high-affinity ligands of rBC2LC-N are the H type 1 trisaccharide (Fuc(1-2)Gal(3)GlcNAc) and H type 3/4 trisaccharide (Fuc(1-2)Gal(3)GlcNAc), corresponding to two forms of the human blood group O determinant. The H type I trisaccharide motifs are present at the end of branched N-linked glycans and O-linked glycans. The exocrine glands of the stomach, duodenum, small intestine, colon and genitourinary tract, presumably associated with secretory processes, i.e., human and mouse, may result in the potential toxicity of LDC in the human body.

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(Continued.) D, Microscopic observation of the resected abdominal wall (skin surface; 1, peritoneum) revealed numerous disseminated cancer cells (arrows) in the control, whereas cancer cells were completely absent after i.p. injection of 1 μg of LDC (H&E staining, scale bar, 500 μm). E, Body weight at day 45 was significantly reduced in the control and rBC2-lectin-without-toxin cohorts (*, P < 0.05; ****, P < 0.0001, two-tailed unpaired Student t test). Intraperitoneal injection of LDC resulted in good weight gain, and body weight was comparable between the i.v. administration and i.p. administration of LDC cohorts. All mice were healthy and did not exhibit damage to noncancerous epithelial cells of hollow or genitourinary organs, and the body weight of the mice was not affected by this treatment. F-4, Intravenous injection of LDC decreased peritoneal dissemination nodules of PDAC in the mouse model. F-2, Laparotomy in the 1 μg i.v. LDC cohort revealed no ascites and the mice appeared slim and normal. F-3, The multiple peritoneal disseminated nodules (arrows) observed in the control. F-4, Laparotomy of controls revealed massive bloody ascites due to active disseminated cancer nodules. F-5, Disseminated nodules at the surface of the liver and nodules at the subdiaphragmatic space (arrows). G, Kaplan–Meier survival curves after LDC treatment in the PDAC dissemination mouse model (n = 9 for each group). The median number of days of survival in the control group (62 days) was significantly improved by i.p. injection of 1 μg of LDC, resulting in 90-day survival of 7/9 mice (median survival = 105 days). Notably, i.v. injection of 1 μg of LDC conferred a comparable effect, with 90-day survival of 5/9 mice (median survival = 90 days; *, P < 0.05; ****, P < 0.0001, n.s., not significant by log-rank test).
setting. Even after i.v. administration, distribution to noncancerous organs should be very limited with the enhanced permeability and retention (EPR) effect, which results in predominant delivery of large molecules to cancer tissues via the damaged endothelial cell linings of capillaries in the region of the tumor (23). In fact, the i.v. injection of 1 μg of LDC/mouse also shrank tumors in the orthotropic (Fig. 4E) and peritoneal dissemination model of Capan-1 cells (Fig. 5F) and subcutaneous PDx tumor (Fig. 4D) with an efficacy identical to that of i.p. injection without notable adverse events. However, it should also be noted that the EPR effect showing the predominant accumulation of LDC in PDx tumors in mouse models may not be reproduced in clinical PDAC, because human PDACs are known to be hypoperfused and poorly vascularized compared with xenotransplanted tumors (24, 25).

Compared with the affinity of antibodies for peptide antigens (Kd = 0.1–10 nmol/L), the affinity of lectins for their target glycans is typically weaker (Kd = 100–1,000 nmol/L). However, LDC possesses the following notable advantages (shown schematically in Fig. 6B): (i) the target glycans are located at the outermost coatings of cancer cells, known as glycocalyx, conferring easy access for targeting drugs; (ii) the cancer cells possess abundant glycan sites for interaction with LDC, including branched glycan chains attached to multiple different core proteins; (iii) the small size of the lectin should be beneficial for drug endocytosis; and (iv) the glycolipids are also the binding target of LDC, and the very close proximity of glycolipids to the cell membrane should be favorable for drug endocytosis. As a comprehensive result, the lectin drug displayed a remarkable IC50 value of 0.0195 pmol/L, nearly 1,000 times lower than the IC50s of conventional immunotoxins, which are on the order of ng/mL (Supplementary Table S2): thus, the lectin drug provides a prominent cancer therapeutic effect. As lectins can be generated in an economical bacterial system, lectin-based drugs should be advantageous from the viewpoint of medical economics in comparison to antibody-based drugs that require costly eukaryotic cells for their production (26).

rB2C2L-C-N may be a unique lectin that lacks typical hemagglutination activity. However, the rapid improvement in lectin engineering technologies (27) will provide control over the harmful adverse effects of some lectins, and synthetic lectin technologies with ideal characteristics as drug carriers. This study provides new insight into cancer treatments that might serve as alternatives to expensive antibody-based drugs. Moreover, the application of tumor-targeting lectins might be expanded by coupling them to small-molecule agents or to nanoparticles, potentially leading to breakthroughs in cancer treatment.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
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