Carbohydrate Mimetic Peptides as Research Reagent and Therapeutic

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Received April 29, 2012

Although numerous carbohydrates play significant roles in mammalian cells, development of carbohydrate-based reagents and therapeutics are hampered by the technical difficulty of chemically synthesizing complex carbohydrate structures. Use of carbohydrate mimetic peptides circumvents this difficulty, as short peptide can be easily synthesized and modified. We as well as others identified carbohydrate mimetic peptides by screening peptide displaying phage library using anti-carbohydrate antibodies and lectins. This review introduces our experiences with I-peptide that was used for identification of new carbohydrate binding receptor expressed in the lung endothelial cells, and those with IF7 peptide that can be used as a therapeutic against malignant tumors.

Key words chemotherapy; SN-38; angiogenesis; annexin; phage display

1. INTRODUCTION

Field of glycobiology has been greatly benefitted by technical advances in genomics and proteomics, as exemplified by cloning of glycosyltransferases and gene knockout mice to determine the in vivo role of specific carbohydrate structure produced by the specific glycosyltransferase. However, due to the lack of automatic synthesis of complex carbohydrates, carbohydrates-based drug discovery has been largely unexplored despite the fact that cancer malignancy is closely associated with carbohydrate structures on the cancer cell surface.1,2) Under the current situation, carbohydrate mimetic peptides identified by phage display library screening provide powerful research tool and reagents.3–5) Furthermore, recent development of tumor targeting carbohydrate mimetic peptide opens a new avenue to glycobiology-based therapeutics.6)

2. IDENTIFICATION OF CARBOHYDRATE MIMETIC PEPTIDES

Several laboratories successfully identified carbohydrate mimetic peptides by screening the peptide-displayed phage libraries.7–10) Many of these successful outcomes were achieved by using monoclonal anti-carbohydrate antibodies and lectins as the target, whereas some used the carbohydrate binding proteins.11,12) We identified a series of linear 7-mer peptides with the consensus sequence IXLLXXR using monoclonal anti-Lewis A antibody (clone 7LE).7) Among them, IELLQAR (isoleucine, glutamic acid, leucine, leucine, glutamine, alanine, arginine), designated as I-peptide, was the best binder to selectins (Fig. 1).

3. INHIBITION OF CARBOHYDRATE-DEPENDENT LUNG COLONIZATION OF CANCER CELLS BY I-PEPTIDE

Many studies suggested that cell surface carbohydrate antigens play important role in cancer metastasis.13,14) Because

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The authors declare no conflict of interest.

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there is no in vitro system to study metastasis, we used an experimental metastasis model with the mouse: cancer cells were injected intravenously through tail vein to colonize the lung, and developed to tumors in the lung. When B16 cells were transfected with FUT3, transfected cells expressed sialyl Lewis X (sLeX) antigen on their cell surface. Intravenously injected sLeX-positive B16 cells colonized the lung more frequently than those untransfected or sLeX-negative B16 cells. We speculated that sLeX-dependent hematogenous lung colonization was mediated by E-selectin and/or P-selectin. However, sLeX-dependent colonization occurred in E-selectin/P-selectin doubly deficient mutant mice, suggesting the existence of yet unknown endothelial receptor distinct from E- and P-selectins. We named such presumptive carbohydrate binding receptor as I-peptide receptor (IPR).

4. IDENTIFICATION OF IPR

When chemically synthesized I-peptide was injected intravenously to a mouse and then sLeX-positive B16 cells were injected, the lung colonization of sLeX-positive B16 cells was significantly inhibited (Fig. 2). This suggested that synthetic I-peptide can be used for affinity matrix for isolating the IPR from the lung endothelial cells. Indeed, synthetic I-peptide conjugated beads enabled us to purify the IPRs (Fig. 3). Proteomics of affinity purified IPR proteins revealed that they are pre-mRNA splicing factors or Sfrs gene products. While identification of pre-mRNA splicing factors as carbohydrate-binding protein was unexpected, this finding parallels observations on galectins and annexins. Galectins are well-established carbohydrate-binding proteins. Galectins are cytoplasmic protein, but they also localize to the cell surface. Furthermore, some galectins localize to the nucleus and exhibit pre-mRNA splicing activity. Similarly, annexins are cytoplasmic protein and some annexin family members localizes to the nucleus and binds to RNA. It is known that annexins bind to carbohydrates including glycosaminoglycans. Although Sfrs, annexins and galectins are distinct genetically, their activities suggest that carbohydrate-binding capacity overlaps with RNA binding-activity.

5. IF7 PEPTIDE AS TUMOR VASCULATURE SPECIFIC TARGETING VEHICLE

During the experiments for identifying IPR, I-peptide affinity chromatography isolated a 15kDa fragment of annexin 1 (Anxa1) (Fig. 3). This suggested that I-peptide binds to Anxa1. Anxa1 was reported to be a highly specific tumor vasculature surface marker in the mouse and human, we looked for a peptide that homes the tumor vasculature but not to the lung. In vivo tumor targeting experiment with phage clones displaying I-peptide related sequences then identified IFLLWQR (isoleucine, phenylalanine, leucine, leucine, tryptophane, glutamine, arginine), designated as IF7, as the tumor specific targeting peptide (Fig. 4).

In Anxa1 null mice, tumor growth was significantly suppressed due to the lack of angiogenesis, suggesting that Anxa1 is essential for tumor angiogenesis. Expression of Anxa1 in the tumor vasculatures is apparently universal to all tumor types in the mouse and in the human, suggesting strongly the therapeutic potential of IF7 for tumor vasculature targeting vehicle.
IF7 peptide was synthesized chemically and conjugated with fluorescent reagent Alexa 488 (A488). When IF7-A488 was injected through the tail vein and tumor fluorescence monitored microscopically, fluorescence signals appeared in the tumor within one minute of injection, reached a plateau in 9 min, and remained high for 40 min6) (Fig. 5). The tumor targeting of IF7-A488 was significantly reduced by anti-Anxa1 antibody that was injected intravenously prior to IF7-A488 injection. These results supported the hypothesis that IF7 targets tumors through Anxa1 expressed on the endothelial cell surface.

6. CHEMOTHERAPEUTICS WITH IF7 PEPTIDE

We asked whether IF7-conjugated anti-cancer drug could suppress tumor growth in vivo. We conjugated IF7 with an apoptosis-inducing anti-cancer drug 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) (GA), a geldanamycin analogue.31) When the IF7-GA was injected intravenously into melanoma, lung carcinoma, prostate cancer and breast cancer model mice, tumor growth was suppressed: tumors from IF7-GA-treated mice were significantly smaller than those from control mice (Fig. 6). It should be noted that the dose of 17-AAG used as IF7-GA was one tenth of 17-AAG used in previous studies of mouse tumor models.32,33) These results indicate that, as proof-of-principle, IF7-conjugated anti-cancer drug suppress tumor growth regardless of tumor types at the dosage lower than that of unconjugated drug.

IF7-GA, however, did not suppress tumor growth completely. We hypothesized that such moderate activity is due to the limited potency of 17-AAG, a drug that induces tumor cell apoptosis by inhibiting Hsp-90.33,34) We therefore conjugated IF7 to a more potent anti-cancer drug, SN-38, which inhibits topoisomerase I.35,36) To ensure the release of SN-38 upon delivery to the tumor, IF7 was conjugated to SN-38 through an esterase cleavable linker37) (Fig. 7). We also added two arginine residues after the cysteine on IF7 to create IFLLWQR-C-RR, or IF7C(RR), to increase conjugate solubility, as IF7C-SN38 was poorly soluble in an aqueous solution. To monitor tumor growth accurately in vivo, we employed luciferase-based cancer imaging. When IF7C(SN38)-SN38 was injected into nude mice bearing large HCT116-Luc tumors, tumor size was dramatically reduced, whereas tumor growth in mice injected with control peptide conjugated SN-38
Fig. 6. Effect of IF7-GA on Melanoma, Lung Carcinoma, Prostate Cancer, and Breast Cancer Models in the Mouse

Size of tumors measured by caliper. (a) Mouse melanoma B16F1 tumors were grown subcutaneously in C57BL6 mice. On day 10, each mouse was injected intravenously with either 100 µL of 5% glucose or that containing 0.13 µmol of each IF7, GA, or IF7-GA. Injections were administered every other day, for a total of three injections, until day 14. Mice were euthanized on day 15 to measure tumor weight. (b) Mouse LLC lung carcinoma tumors were grown subcutaneously in C57BL6 mice. On day 7, each mouse was injected intravenously with the compounds as in (a), and injections administered every other day, for a total of three injections, until day 11. Mice were euthanized on day 13 and tumors weighed. (c) Human prostate cancer PC3 tumors were grown orthotopically in the prostate of SCID mice. On day 7, each mouse was injected intravenously with the compounds as in (a), and injections administered every 4 d, for a total of four injections, until day 22. Mice were euthanized on day 28 and tumors weighed. (d) Human breast cancer MDA-MB-231 tumors were grown orthotopically in fat pads of SCID mice. On day 7, each mouse was injected intravenously with the compounds as in (a), and injections performed every 4 d, for a total of four injections, until day 22. Mice were euthanized on day 28 and tumors weighed. Asterisks show statistical significance (Mann–Whitney’s U-test).

Fig. 7. Conjugation of IF7C(RR) with SN-38

Conjugation of IF7C(RR) with SN-38 followed the method described by Meyer-Losic et al. with modifications. Tranexamic acid was conjugated with heterofunctional cross-linker succinimidyl-4-((N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), which was then esterified with SN-38. SMCC-SN38 was then conjugated with IF7C(RR) peptide through maleimide-and sulfhydryl linkage. The product IF7C(RR)-SN38 was purified by using a C18 reverse-phase HPLC column in HPLC by elution in a linear gradient from 50% to 70% acetonitrile in water containing 0.01% trifluoro acetic acid over 20 min. The structure of the purified product was assessed by ESI mass spectrometry.
continued (Fig. 8). It is noteworthy that, despite the large tumor size, the dosage of IF7C(RR)-SN38 administered was much lower than those used in a previous study. Histological examination of tumors treated with IF7C(RR)-SN38 revealed significant necrosis and apoptosis. Blood tests showed no sign of side effects in peptide-SN38-injected mice.

7. CARBOHYDRATE-BINDING ACTIVITY OF ANXA1

Annexins exhibit an evolutionally conserved core domain and N-termini unique to each family member. All annexin family proteins bind to phospholipids in a calcium dependent manner. Several annexin family proteins bind to sulfated glycosaminoglycans suggesting that sulfated glycosaminoglycans bind to conserved domain of annexins.

Glycan array analysis showed that Anxa1-His6 protein bound to a series of fucosylated glycans. LeA-PAA (Lewis A oligosaccharide conjugated to polyacrylamide) bound to Anxa1-His6 protein in a plate assay. IF7 peptide inhibited LeA-PAA binding to Anxa1-His6, suggesting that both fucosylated glycans and IF7 share the binding site in Anxa1. Since an anti-Anxa1 antibody raised against N-terminal domain of Anxa1 inhibited IF7 binding to Anxa1 (Fig. 5), it is likely that IF7 binds to N-terminal region of Anxa1 of which sequence is unique to this protein. IF7 did not bind to a deletion mutant of Anxa1 protein, lacking N-terminal 12 amino acids. Future studies should define the structural basis of Anxa1 for binding to fucosylated glycans and IF7.

8. PERSPECTIVE OF IF7-CONJUGATED DRUGS

Since the N-terminal domain of Anxa1 is completely conserved in the mouse and human, we anticipate that IF7 would bind to human Anxa1 expressed on endothelial cell surface in the tumors in humans. Given the difficulty of chemically synthesizing complex oligosaccharides, carbohydrate mimetic peptides provide us with an excellent alternative. Although the peptides are susceptible to proteases, in the case of IF7 the intended role of IF7 is as a drug delivery vehicle. Peptide moiety of drug should remain intact until it reaches the tumor vasculature, where it can then be degraded. We speculate that at least some, if not all, IF7-conjugated drug is delivered to the tumor before proteolysis occurs, since IF7-GA and IF7C(RR)-SN38 exhibited anti-tumor activities at considerably lower doses than peptide-SN38.
lower doses than those required without IF7 (Figs. 6, 8).

The efficacy of a peptide-conjugated anti cancer drug also depends on the chemistry of conjugation. We used an esterase-resistant linker for GA and an esterase-cleavable linker for SN-38. When IF7C(RR)-SN38 was incubated at 37°C with mouse plasma, as much as 50% of SN-38 was released from the conjugate within 10 min. As it takes 9 min for IF7 to target the tumor (Fig. 5), this suggests that tumor growth suppression was achieved by IF7C(RR)-SN38 survived in the initial 10 min window upon intravenous injection. Although IF7C(RR)-SN38 may be more stable in human plasma, which exhibits weaker esterase activity than does mouse plasma, future studies should address ways to both enhance drug stability in the circulation and promote efficient drug release in tumor tissue.

We have explored therapeutic potential of Anxa1-binding peptide as a cancer treatment. The extremely efficient tumor vasculature targeting activity by IF7 and possibility for further improvement of this technology warrant evaluation of clinical efficacy of anti-cancer drugs conjugated to Anxa1-binding peptides in patients.

REFERENCES


