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Improving Theranostics in Pancreatic Cancer

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Abstract

Background—Pancreatic cancer is the fourth most deadly cancer in the US, and is expected to be the second most deadly by 2030. The major difficulty in treating pancreatic cancer is the late onset of symptoms. Generally, patients show metastatic disease by the time of diagnosis, with a survival rate of 5% beyond 5 years. In patients without metastatic disease, surgical resection increases 5 year survival rate to 25%. The remaining 75% succumb to undetected metastases. Clearly, improvements to both detection, surgical intervention, and therapeutic strategies will be needed to improve patient outcome in pancreatic cancer.

Methods—Recent literature has been surveyed and atomic models of new therapeutic approaches were generated.

Results and Conclusions—Here, we focus on the recent progress employing monoclonal antibodies (mAbs) to target pancreatic cancer associated markers, and more specifically on recent chemical and protein engineering efforts to improve the homogeneity, stability, and administration of mAbs to precisely delivery imaging agents and cytotoxins to sites of disease.

Keywords

Antibody Engineering; Site Directed Conjugation; Theranostics

Introduction

Monoclonal antibodies can be generated with extraordinary specificity and pharmacological properties. They are frequently used to block signaling, to down regulate surface receptors and to elicit an immune response. Their specificity has been further exploited to deliver potent cytotoxins, leading to cell death. While the goal of these antibody-drug conjugates (ADCs) is to deliver potent cytotoxins directly to the diseased cell and kill it, thereby avoiding healthy tissues, additional benefits have been observed (1). These benefits include bystander effects where the processed drug is released from the lysed cell and kills adjacent tumor cells. ADCs also have been described to potentiate ‘immunogenic cell death,’ where

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potent immune stimulators such as Damage-Associated Molecular Patterns (DAMPs) and cytokines released by the targeted cell attract T cells (2). Beyond the delivery of cytotoxins, the exquisite specificity of mAbs can also be used for *in vivo* immunohistochemistry, carrying PET and/or SPECT imaging agents (ref). They can also be used to carry optical markers and are currently being developed for intra-operative optical imaging (IOOI) (3). Ultimately, the success of this approach relies on the disease target, the PK/PD of the imaging agent including homogeneity, and the chemistry (coupling of the agent to the antibody) and the physics (half-life of the reagent) of the imaging agent. Herein, we review each of these aspects with an emphasis on pancreatic cancer targets.

Methods

Recent literature has been surveyed and atomic models of new therapeutic approaches were generated using PyMol (4) and structures deposited in the PDB (5). We only focus on antibodies and representative examples. Numerous excellent reviews on different aspects of protein engineering, conjugation and applications are available and update frequently.

Results

Targets in pancreatic cancer

Only a handful of useful markers for pancreatic cancer are known, and being explored for theranostic applications. The main targets are CEA, CA19.9, MUC1, and TAG72, which are predominantly aberrantly glycosylated proteins (Fig. 1). A large number of additional mAbs are being developed including mesothelin, ROR1, Tissue Factor (TF) and others.

CEA—Carcinoembryonic antigen (CEA/CEACAM5) is a glycoprotein consisting of three repeats domains attached to the cell surface of enterocytes by a GPI anchor. The proposed role of CEA is to function in cell adhesion and cell signaling (6). In normal adults, CEA is expressed on the luminal surface of epithelial cells, whereas in neoplastic tissue expression is on both the luminal and basolateral surfaces. Antibodies are unable to access luminal CEA, preventing antibody binding to normal tissue. CEA expression is found in many tumors, including pancreatic. Several monoclonal antibodies have been developed against CEA, such as labetuzumab, KAb201, and M5A(7–9) CEA undergoes a conformational change when shed that is not recognized by some antibodies(10). This allows for specific targeting of cytotoxic agents to cells expressing CEA.

CA19.9—CA19.9, sialyl lewis A, is a carbohydrate antigen expressed on many tumor tissues in the gastrointestinal tract (11). Like CEA, it is overexpressed in the majority (>90%) of people with pancreatic cancer (12). CA19.9 is expressed at low levels in normal pancreatic tissue. Instead, healthy epithelial cells express disialyl lewis A (13). The stark difference in expression levels between healthy and cancerous tissue makes CA19.9 a great marker for pancreatic cancer. Indeed, CA19.9 released into the serum is currently used as a diagnostic for pancreatic cancer, with several monoclonal antibodies for monitoring CA19.9 levels having been developed (14, 15). Unlike CEA antibodies, CA19.9 antibodies cannot distinguish between serum and tumor localized antigen, making CA19.9 a poor candidate

for cytotoxic targeting (15). However, CA19.9 remains useful for diagnosing and monitoring pancreatic cancer.

Mucin1—(muc1) is a large membrane-bound glycoprotein located on the apical surface of pancreatic epithelial cells. Muc1 consists of a series of extracellular 20 amino acid repeat domains, a transmembrane domain, and a cytoplasmic domain (16, 17). Alternative splicing results in different isoforms of muc1, with differing numbers of the repeat domain. In healthy tissue, muc1 is restricted to the apical surface of the epithelial cells (18). In pancreatic cancer, epithelial cells lose their polarity, resulting in muc1 expression in the basolateral membrane (18). In most pancreatic cancers (90%), muc1 is overexpressed and hypoglycosylated (19). The change in location and expression levels have made muc1 a target for immunotherapy. Several clinical antibodies have been developed for muc1: HMFG, PankoMab, and cantuzumab (20, 21). HMFG and PankoMab recognize a peptide motif (PDTR) in the repeat domain (21). PankoMab recognizes the glycosylated form of the peptide (22). Both antibodies have been humanized. HuHMFG is currently being explored for breast cancer treatment. Cantuzumab binds a tumor-specific glycoform of muc1 (23). An additional antibody, PAM4, was thought to target muc1, but was later determined to target MUC5AC (24).

TAG72—Tumor-associated glycoprotein (TAG)-72 is a mucin-like glycoprotein present in many cancers including pancreatic, colorectal, gastric, and ovarian cancers (25, 26). The antigenicity of TAG-72 arises from abnormal glycosylation of proteins with the carbohydrate Sialyl-Tn (27). In healthy adults, sialyl-Tn levels are low and localized to specific cell types (28). Sialyl-Tn is not normally exposed to the immune system. Like other pancreatic markers, TAG-72 is often shed into the serum of diseased individuals. Antibodies against TAG-72 were originally developed against membrane fractions from human breast carcinoma cells. Second generation antibodies against TAG-72 were made using immunopurified TAG72 (29). An alternative approach for generating TAG-72 antibodies was to immunize mice against ovine submaxillary mucin, which contains sialyl-Tn as 90% of its carbohydrate content (27).

Mesothelin—Mesothelin is a 40 kDa GPI anchored protein, normally expressed in mesothelial cells (30). The function of mesothelin is unknown, but it has been shown to interact with MUC16/CA125 (31). It is expressed as a 71 kDa precursor, and processed to generate the membrane bound mesothelin protein and the soluble megakaryocyte potentiating factor (30, 32). It is overexpressed in mesotheliomas, pancreatic, ovarian, and lung cancers (33). The high level of expression in tumors has made mesothelin a target for immunotherapies. The most well-characterized antibodies against mesothelin are SS1 and MORAb-009 (34). SS1 is an affinity-improved dsFv derived from a murine mAb (35). An immunotoxin version of SS1 has been tested in clinical trials for mesothelin expressing tumors (33). MORAb-009, also known as Amatuximab, is a chimeric antibody developed from SS1 (36). MORAb-009 and SS1 bind to the same region of mesothelin as MUC16. Recently, additional mAbs have been developed which bind to alternative regions of the mesothelin protein, allowing for additional mesothelin targeting in the presence of MORAb-009/SS1 or MUC16 (37).

Functionalization of mAbs for Delivery of Toxins and Imaging Agents

Monoclonal antibodies are frequently described as magic bullets – highly specific and capable of delivering a lethal punch. They are readily generated through immunization and hybridoma technologies, and more recently, through display techniques (e.g., yeast display). Combination of modern molecular biology techniques and elegant selection methods have led to impressive gains in specificity, affinity and stability. As such, hundreds of mAbs are being developed to target multiple forms of cancer and other diseases. In some cases, mAbs are capable of killing tumor cells alone, either through down regulation of tumor associated receptors, inhibiting signaling pathways that drive tumorigenesis or evoking an immune response such as antibody dependent cell-mediated cytotoxicity. When these fail, mAbs can be used to target potent cytotoxins to the tumor. Alternatively, they can be used to mark tumors, not only to identify the tumor but also for surgical resection. Adding such functionality to mAbs with precision has been a challenge until recently (38).

Direct conjugation—Until recently, the ability to functionalize mAbs relied upon direct conjugation through reactive groups of natural amino acid side chains present on the surface of the mAbs (Fig 2). A major issue with this approach is the combinatorial nature of the conjugation. There are a large number of lysines on the surface of mAbs, each capable of reacting with imaging/therapeutic agents conjugated through N-hydroxysuccinimide esters (NHS) or other amine reactive coupling chemistries (fig). Mass spectrometry studies of Trastuzumab emtansine (T-DM1) have identified multiple species ranging from no toxins up to seven toxins (39). Assuming each amine is equivalent, this would lead to 128 different combinations (e.g., no conjugates, seven single conjugates, twenty one double conjugates, etc...). While T-DM1 is in the clinic and proven to provide clinical benefit, it is widely acknowledged that the heterogeneous distribution affects the PK and PD (40).

Conjugation to cysteines provides an alternative chemistry for limited labeling of an antibody. The surface exposed disulfides found in the hinge region of the chain and those found between the light and heavy chains in Fab region can be gently reduced for conjugation. This reduction results in a maximum of 8 sites for conjugation, and limits the combinatorics. Early studies used maleimide chemistry for the cysteine conjugation (Fig. 3). A significant concern with this approach is that the maleimide conjugate blocks the reformation of the disulfide and destabilizes the IgG, which would negatively affect its PK.

More recently, several elegant chemical approaches have emerged to exploit these cysteines but retain the covalent nature of the original disulfide bonds (Fig. 3). Specifically, bis-sulfones react with both sulfurs of the reduced cysteines, effectively replacing the disulfide with a three carbon bridge (41, 42). Dibromomaleimide is an alternative approach to insert a two-carbon bridge across disulfide (43, 44). In both cases, functionality (denoted R in Fig. 3) is introduced through the alkylating agent. Nearly stoichiometric coupling was observed using PEGylated bis-sulfones (42) whereas a six-fold excess was needed to conjugate monomethyl auristatin E to the mAb (45).

In addition, the glycosylation, predominantly within the Fc region, opens up the possibility of conjugating directly to the sugar moieties. Senter and his group at Seattle Genetics have added a large number of fucose analogs into the culture media and found a number of these

analogs were incorporated into the glycosylated mAb including thiol and azide derivatives (46). Using a thiol derivative, they achieved ~70% incorporation of the thiol glycan and subsequently added ~1.3 toxins to the mAb. The homogeneity and the stability of the resulting mAb conjugate was significantly improved over conjugation methods to the hinge disulfides (46). Similarly, Boons and his group have incorporated azides into the glycan structure through galactosyltransferase (47). They achieved a higher level of incorporation of an azide handle and a higher level of drug conjugate, ~3.5. Others have followed a similar strategy with success (27882199). Alternatively, the Schibli group(48)and subsequently others (40) have used transglutaminase to tether lysine-bearing imaging agents and cytotoxins to mAbs. First, the mAb must be deglycosylated, and as with other strategies, the ligand is added at high molar ratios to the mAb (upwards of 80 to 1).

Finally, Tourkas and his group achieved site specific labeling using IgG binding proteins. They incorporated a photoreactive amino acid, benzoyl-phenylalanine, into the IgG binding domains of proteins G or A (Fig. 4). A second unique amino acid is also incorporated into proteins G or A for subsequent conjugations (49, 50). Exposure to UV light, 365 nm, activates the benzoyl-phenylalanine which goes on to form covalent bond to the bound mAb. Because there is a natural affinity for the mAb, the ratio of ligand to Mab is closer to unity (8 to 1). Using this method, they have successfully added PEGs, biotin, fluorescent markers, etc. to a number of unmodified IgGs, including cetuximab and Okt3, an anti-CD3 mAb.

Protein Engineering—The issue of heterogeneous mixtures due to random conjugation to naturally occurring residues has led to extensive protein engineering efforts. These include the incorporation of non-natural amino acids, substitution of surface residues to cysteine or seleno-cysteine at select position and the incorporation of peptide substrates that can be post-translationally modified.

Incorporation of non-natural amino acids—The redundancy of the genetic code has allowed investigators to use orthogonal pairs of an aminoacyl-tRNA synthetase and tRNAs to incorporate non-natural amino acids in to proteins (51, 52). P-acetyl-l-phenylalanine has been placed at multiple positions of mAbs allowing for the efficient conjugation of toxins, imaging agents and other biologics (53, 54). Recently, the Schultz group demonstrated the incorporation of two orthogonal aminoacyl-tRNA synthetase and tRNA pairs in a mammalian cell line opening up the possibility of adding imaging and cytotoxins at different sites (55). A similar approach has been implemented using cell-free synthetic approaches (56).

Junutula and his team at Genentech developed an elegant cysteine scanning method to on the surface of mAbs to identify potential sites to place a cysteine that are amendable to chemical modification (57). This scan afforded a number of sites with variable reactivity. Of these, only a handful was further developed. Follow up studies indicated that the location of the cysteine was critical to the stability and efficacy. Substitution at V205C in the heavy chain produced the most favorable results in pre-clinical studies (58), only requiring a three-fold excess of the maleimide-cytotoxin (Fig. 5)

Substrates and tags for conjugation—Bertozzi and her team recognized that formylglycine generating enzyme (FGE) could be used to convert the cysteine residue in the sequence CxPxR to an aldehyde for labelling with amonooxy- or hydrazide reagents (59–63). Fusing this sequence to the end of the mAb Fc allowed the efficient addition of potent cytotoxins and imaging agents (Fig. 6). Placing this sequence within the Fc permitted efficient addition of different glycosylation analogs(64).

Similarly, Geierstanger and his group recognized that phosphopantetheinyl transferases (PPT) are capable of functionalizing mAbs containing PPT substrate sequences (65). Inserting the PPT substrate sequence at over 100 sites, they identified several sites that were amenable to the PPT modification. Using a ~ 10 to 40 fold excess of toxin conjugated, coenzyme A, they achieved over a 90% yield of doubly labeled mAbs in the presence of PPT (65).

Ligases have also been exploited to add new functionality to mAbs. These include Sortase (66), tubulin tyrosine ligase (67), and spyligase (68). In each case a specific peptide sequence is added to the C-terminus of the light and/or heavy chains of the mAb. A second peptide sequence, that is readily synthesized by solid phase peptide synthesis, is then functionalized with the target drug or carries a unique tag that can be subsequently functionalized. The tagged mAb and functionalized peptide are then joined together in the presence of the ligase. As before, the imaging agent or cytotoxin is typically added at 20 fold or more excess (66).

Applications

The high specificity and the numerous strategies that have been developed as described above have put mAbs front and center in the push for precision medicine.

Imaging—An immediate application is their application in *in vivo* immunohistochemistry. Mortimer and Raubitschek and their colleagues have conjugated DOTA to trastuzumab and used ⁶⁴Cu to identify Her2+ metastatic lesions in breast cancer patients (69). Others are developing similar approaches for other targets, often with the goal of radioimmunotherapy (70). Beyond the issue of tumor penetration (71), a major challenge with using mAbs to image disease is the discrepancy between amount of time it takes for an antibody to accumulate at the disease site (signal) and clear the blood stream (noise) as well as the half life of the radionuclide. Moreover, a circulating radiolabeled antibody will deposit significant amounts of radiation to healthy tissues. Pretargeted imaging methods have evolved to circumvent this issue (Fig. 7) (72–74). Originally described in 1988, the general principle is to separate the two processes. By creating antibodies against haptens that chelate radioactive nuclides, it was possible to administer these antibodies and allow them to accumulate at the tumor (non-specifically) and clear from the blood stream (74). The radioactive hapten can then be administered. The hapten rapidly binds the antibody at the tumor and unbound hapten is eliminated by renal filtration. This allowed the investigators to image at a much earlier time point post administration of the radionuclide, reducing the number of half-lives while mitigating the background signal from mAbs in circulation. This approach also reduced ionizing radiation to healthy tissues.

Protein engineering efforts have significantly improved this approach. First, the creation of bispecific antibodies allowed one Fab arm to specifically target tumor-associated antigen and the other arm to bind to a radioactive antigen (Fig. 8) (75). Moreover, it is possible to use a bivalent (76) or even multivalent haptens (77) to increase the specificity and affinity through avidity. Second, it is possible to add an orthogonal group to the mAb (through methods described above) and directly react a radioligand to this group in vivo (78, 79). Click chemistry using strained octynes and azides is one possibility, however, the reaction rate is too slow for pretargeted imaging. Rather, a much more rapid reaction, identified and developed by Fox and his group (80) and Robillard and his group (81), uses the Inverse-electron-demand Diels–Alder reaction. Of particular interest is the ability to combine this approach with ^{18}F for PET imaging (82).

Finally, through our own efforts, we recently identified a unique binding site for a cyclic amino acid peptide within the Fab arm of the monoclonal antibody Cetuximab (83). We have demonstrated that this site is absent in human mAbs, but can be readily grafted on to other human mAbs including trastuzumab. We have also demonstrated that we can use multivalent mediotopes to specifically target tumor associated antigens pre-bound with mediotope-enabled mAbs (83). Initial imaging studies using mediotope-enabled Tag72 mAb in animal tumor xenografts are promising and will soon be reported.

Translation of the early incarnations of the pretargeted approach has entered preclinical trials and has yet to make it to the clinic. Several clinical studies on pretargeted imaging and pretargeted radioimmunotherapy using CEA have been conducted on non-pancreatic cancers. A phase I/II clinical trial using pretargeted radioimmunotherapy with an anti-CEA $\times$ anti-hapten on medullary thyroid cancer showed positive results on smaller tumors (<37 mm in the greatest diameter) (84). An additional phase II clinical trial on medullary thyroid carcinoma using a different anti-CEA antibody also found positive antitumor activity with manageable toxicity (85). A phase I clinical trial using the dock-and-lock technology (anti-CEA $\times$ anti-histamine-succinyl-glycine (HSG)-humanized trivalent bispecific antibody) for pretargeting against lung cancer was performed to determine dosage of antibody and label. Interestingly, they found that a pretherapy imaging session was good indicator of absorbed doses during therapy sessions (86). The latest targeting moieties and chemical approaches, however, have demonstrated impressive results in animal models, establishing promise for the eventual application in the clinic.

Fluorescence-guided surgery—Another potent application of mAbs is to define adequate margins in complex tumor resections. Tumor resection studies in animal models using tumor targeting mAbs that are conjugated to fluoroscope compatible dyes have allowed submillimeter lesions to be laparoscopically resected (87). This approach leads to higher survival rates compare the to same operation but only using a bright light source (87). This approach can be further combined with PET imaging to provide a roadmap before the surgical procedure and subsequently use for determining margins (88).

Conclusions

The accumulation of advances in protein engineering, conjugation chemistry, and imaging and surgical technologies is beginning to bear fruit and the future of treating and curing pancreatic cancer is bright.

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Synopsis

Recent advances in molecular biology, protein engineering, and chemical synthesis are enabling in vivo immunohistochemistry and making significant advances in precision medicine. A review and a molecular account is presented.

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Figure 1.
Target antigens for pancreatic cancer

Figure 2. General Structure and reactive side chains in monoclonal Antibodies

A) Monoclonal antibodies are comprised of two polypeptide chains, a heavy chain (white surface) and the light chain (light blue surface). The light chains pair with the heavy chain, creating the Fab region, which is responsible for targeting antigen (at the CDR loops). The heavy chains dimerize creating the Fc region, which mediates an immune response. The two notable natural occurring amino acids that are amendable to chemical conjugation are lysines (blue patches) and cysteines (orange patches). **B)** While the lysines can be directly modified through NHS and other amine specific chemistries, the conjugation to the cysteine residues requires gentle reduction to break the disulfide bonds to free the thiols.

Figure 3. Functionalization through cysteines

Upon reducing the disulfides, there are a number of reagents that react with the free thiol. A) Maleimide has been extensively used to conjugate to free cysteines within proteins. An issue with this coupling reagent, however, is the loss of the disulfide bridge between the different subunits and the subsequent instability that occurs. This issue has been recently remedied by the use of bis-functional linkers that effectively re-bridge the different subunits. These include the reagents shown in B) – D). Bis-sulfone (B) inserts three carbons between the disulfide whereas the dibromomaleimide and the dibromopyridazinedione only add two. Emanating from each linking moiety is the drug/imaging agent (denoted R). Frequently, between the imaging agent and the reactive group, are polyethylene glycol (PEG) subunits, ranging from four to twenty five subunits. Modeled to scale on an IgG are three dolastatins bridged by fifteen subunit PEG. While the PEG bridge is highly flexible, it is clear that these functional groups are not necessary minor perturbations to the mAb.

Figure 4. Ligation through photochemistry

Beyond directly conjugating to natural amino acids, it is also possible to use IgG binding proteins to juxtapose a photo-reactive group to the IgG and use light to crosslink the two molecules. Imaging agents can be incorporated within protein G, either through sortase or other ligases, or through the introduction of non-natural amino acids (e.g., azido bearing amino acids).

Figure 5. Protein engineering and site-specific conjugation

The introduction of unique residues within the IgG mitigates the issue of heterogeneous mixtures. Through extensive screening a number of positions within the IgG framework were amendable to the substitution of a cysteine. Shown here are two positions for site-specific conjugation on trastuzumab, one on the light chains and one on the heavy chains of the Fab. Though near the CDR loops, the drug conjugates sufficiently distant to sterically interfere from antigen (Her2) binding. Using unique tRNAs, it is also possible to substitute non-natural amino acids within the antibody framework and achieve the same level of labeling homogeneity.

Figure 6. Post-translational modification

In addition to substituting unique amino acids, it is also possible to encode substrate sequences for ligases and other post-translation modification. The five residue substrate for the FGE enzyme has been successfully placed within the Fc region as well as the C-terminal tail. Exposure to FGE, either co-expressed or in vitro, converts the substrate cysteine to a reactive aldehyde. Alternatively, the substrate for sortase ligase and tyrosine tubulin ligase can be genetically fused to the C-termini similar to the FGE sequence and used to ligate toxin and imaging bearing ligands.

Figure 7. Pretargeted imaging

A) Schematic of pretargeted imaging. B) A comparison of radioactivity levels between traditional imaging and C) pretargeted imaging. In pretargeting imaging, the level of radioactivity is higher at the time of imaging. Additionally, the unbound radioactive label is rapidly cleared in pretargeted imaging.

Figure 8. Protein engineering to improve pretargeted imaging

A) Bispecific mAbs - one arm binds to the tumor antigen and the other binds to low molecular weight hapten. B) Pretargeting with trans-cyclooctene conjugated-mAbs for tracing with radiolabeled tetrazine C) Pretargeting with mediotope-enabled mAbs for tracing with radiolabeled mediotope.