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DRUG TARGETING WITH MONOCLONAL ANTIBODIES

A review

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Monoclonal antibodies have been widely used in attempts to target anti-neoplastic agents selectively to tumours. Problems associated with the use of monoclonal antibodies as the targeting moiety include lack of complete tumour selectivity, antigenic heterogeneity, tumour access and immunogenicity. Considerable effort in the targeting field is being expended in an attempt to reduce or overcome these problems. Attachment of monoclonal antibodies to low molecular weight cytotoxic drugs, protein toxins, radionuclides or enzymes capable of conversion of inactive prodrugs to cytotoxic drugs, has, despite these problems, resulted in conjugates which do have selective anti-tumour effects in animal models. The advantages and limitations of these different approaches are reviewed. It remains to be established in man if any of these approaches will result in significant therapeutic benefit in major solid tumours.

Key words: Neoplasms/therapy, anti-neoplastic agents, tumour antibodies, antibody-toxin conjugates, antibody-drug conjugates, antibody-radionuclide conjugates.

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Systemic administration of low molecular weight anti-neoplastic drugs to patients with solid tumours results in the preferential distribution of the drug in normal tissues rather than in the tumour since most solid human tumours are poorly vascularised compared to most normal tissues (1). The selectivity of many anti-neoplastic drugs in clinical use therefore relies on the fact that they preferentially kill proliferating cells. There are, however, two major drawbacks in using this as a basis for selectivity. Firstly, normal tissues (bone marrow, gastrointestinal tract) have proliferating cell populations and the destruction of these leads to dose limiting toxicity with most anti-neoplastic drugs in clinical use. Secondly, the fraction of proliferating cells at any time in most solid tumours is often extremely low (2) and thus such agents only kill a small percentage of the tumour cells. Repeated administration of these agents is therefore required to kill a significant number of the tumour cells.

The aim of drug targeting is to localise selectively anti-neoplastic agents at the tumour site and thus spare normal tissues from harm. Such selective localisation should allow the use of agents which can kill both the proliferating and quiescent populations in a tumour. The idea of drug targeting was first put forward over 80 years ago by Paul Ehrlich. He described the concept of a 'heptophore' which carried a 'toxophore' selectively to a tumour. Initial attempts to put this concept into practice using a variety of targeting agents as the 'heptophore' including polyclonal anti-sera, while demonstrating the feasibility of this concept in experimental models, have had little clinical impact. The advent of hybridoma technology in the 1970s has resulted in the generation of a large number of monoclonal antibodies directed against cell surface antigens selectively expressed on tumour cells. This has led to an upsurge in interest in drug targeting using monoclonal antibodies.

In this review the use of monoclonal antibodies as the 'heptophore' will be discussed and different antibody-based targeting strategies will be reviewed, emphasis being placed on the advantages and limitations of each approach. The reader is referred to a number of excellent recent reviews for more detailed information on the different approaches (3–8).

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Tumour specific monoclonal antibodies

The majority of specific monoclonal antibodies reacting to human tumours have been produced by immunising mice with preparations of tumour cell lines or human tumours. Hybridomas secreting monoclonal antibodies are produced by fusing B-lymphocytes from such immunised mice, with malignant plasma cells called myelomas. The immortalised hybridoma cells, once separated from unfused B-lymphocytes and myeloma cells, are screened to identify individual hybridoma clones which secrete a tumour reactive monoclonal antibody recognising a tumour cell antigen. To identify tumour-specific monoclonal antibodies the resultant hybridomas secreting tumour reactive monoclonal antibodies are screened against normal human tissues. Many hybridomas from a single splenic fusion are likely to be tumour reactive but few, if any, will be tumour selective since the antigens with which they react are likely to be present on both tumour and normal tissues.

Tumour-associated antigens can arise, however, for a number of reasons (9). Firstly, cell surface antigens on normal cells may be overexpressed on the tumour cells. For example, leukaemic cells induced by the human T-cell leukaemia virus, HTLV-1, have 10- to 100-fold more surface receptors for interleukin-2 than do normal T-lymphocytes (10). Secondly, malignant cells tend to become more primitive and as a result express 'oncofoetal' antigens normally associated with foetal cells of the same cell lineage as the tumour cell. Examples of such antigens include carcinoembryonic antigen (CEA) and placental alkaline phosphatase (PLAP). Thirdly, antigens associated with proliferation, such as the transferrin receptor, are often expressed at higher levels on tumour cells (11). Fourthly, tumour cells, due to defects in their glycosylation pathways, often express unusual carbohydrates on both glycoprotein and glycolipid components of their cell membrane. Fifthly, structural variants of normal proteins can be produced due to mutations in the DNA of the cell. An example of this is the p97 antigen associated with melanoma.

Monoclonal antibodies reactive with all these antigens may cross-react with normal tissues since the normal tissue expresses the same antigen or a structurally related molecule. Some of the best characterised and most widely used tumour selective antibodies, HMFG1, B72.3, 17-1A, all react with certain normal tissues (8, 9). When tumour selective monoclonal antibodies are attached to potent cytotoxic agents the consequences of this cross-reactivity in terms of toxicity to normal tissues can be profound (see below). It thus remains a major goal in the targeting field to identify new tumour selective monoclonal antibodies with minimal or no normal tissue cross-reactivity.

An alternative strategy for targeting tumours with monoclonal antibodies has recently been reported (12). It involves using antibodies directed against insoluble intracellular antigens such as histones, which are not accessible to the antibody in viable cells of normal tissues and tumours. However, in solid tumours a common feature is the presence of areas of necrotic cells due to poor vascularisation. The membranes of these necrotic cells are permeable and thus the antibodies directed against intracellular antigens can gain access to them. Successful tumour imaging using such antibodies has been demonstrated both in rodent models and in patients. It remains to be determined whether such antibodies can be used for drug targeting.

Apart from normal tissue cross-reactivity, a number of additional problems exist which impact on the use of monoclonal antibodies as the 'heptophore'. Monoclonal antibodies produced from B-lymphocytes of mice will be immunogenic in man since they contain foreign epitopes. Once an immunogenic response has occurred, subsequent doses of the antibody or conjugate will be rapidly removed from the blood stream by the neutralising antibody and thus tumour localisation will be impaired. Production of human monoclonal antibodies using human B-lymphocytes has to date proved difficult mainly due to instability of the hybridomas. An alternative strategy has been to use genetic engineering techniques to create 'humanised' mouse monoclonal antibodies (13). These contain the antigen-binding variable region domain of the mouse antibody and the framework constant region domain of a human antibody. In the clinic it has been found that the humanised version of the mouse monoclonal antibody 17-1A, which reacts with colon carcinomas, was completely non-immunogenic in 9/10 patients even after multiple administrations (14).

Monoclonal antibodies are large molecules (150 000 Da) and tend to localise to tumour cells adjacent to the blood capillaries. Tumour penetration of such large molecules is limited by a number of factors including the high interstitial pressure in tumours and the poor vascularisation (1). In an attempt to improve penetration, antibody fragments have been generated both by enzymic and more recently by recombinant techniques (15). Enzymatically generated antibody fragments have been shown to penetrate tumours more rapidly than their intact antibody counterparts. However, since the antibody fragments are removed much more rapidly from the bloodstream than their intact antibody counterparts, the percentage of the injected dose reaching the tumour is smaller for fragments than for intact antibody (16). It thus still remains to be resolved whether intact antibody or an antibody fragment is the optimal 'heptophore' for drug targeting.

Most antibodies recognise antigens which are expressed heterogeneously in the tumour cell population. Consequently, a proportion of cells within the tumour will not bind the antibody. If the therapeutic strategy requires direct binding of the antibody to the target cell this could represent a major problem. Possible ways to overcome this problem are to use a cocktail of monoclonal antibodies

reactive with a number of different tumour-associated antigens or by combining the therapy with another therapeutic approach which does not rely on the antigen expression for its therapeutic effect. Alternatively, agents such as α-interferon have been used which have been found to upregulate the expression of certain tumour-associated antigens and so reduce the number of antigen negative-tumour cells (17).

Cytotoxic drug conjugates

A wide range of low molecular weight anti-neoplastic cytotoxic drugs including methotrexate, vinblastin, daunomycin and melphalan have been attached to monoclonal antibodies in an attempt to deliver them selectively to tumours and thus overcome the toxicity problems associated with these drugs (3, 5). A number of different conjugation strategies have been investigated, the aim of which are to introduce the maximum number of drug molecules per antibody under conditions where both antibody-binding reactivity and drug activity are retained. These strategies include a) covalent attachment of the drug by direct chemical linkage of the drug to amino acids or carbohydrate residues in the antibody, b) using small spacer molecules such as an acid cleavable cis-aconityl spacer or an oligopeptide spacer cleavable by lysosomal enzymes, as a linker between drug and antibody, and c) attachment of the drug either directly or via a spacer molecule to a high molecular weight carrier molecule such as albumin, poly-L-lysine or dextran which is then attached to the antibody. Optimisation of conjugation is important for two reasons; firstly to introduce the maximum number of drug molecules per antibody molecule because many thousands of these drug molecules have to be delivered to each tumour cell to exert a cytotoxic effect and secondly to allow release of free active drug within the cell, since conjugates in which drug is not released have reduced potency compared to the free drug. Thus, cell killing with most cytotoxic drug conjugates involves initial attachment of the conjugate to the cell surface, antigen-mediated internalisation and release of free drug in the lysosomal compartment of the cell.

While use of a high molecular weight carrier results in the greatest number of drug molecules per antibody molecule the resulting conjugates are often extremely large and thus the tumour access problem (see above) is exacerbated. In addition, the pharmacokinetics of these conjugates in vivo may be compromised, rapid entrapment of antibody-human serum albumin-methotrexate conjugates being reported in the liver in mice. Despite the problems described above, antibody-drug conjugates have been found to exert superior anti-tumour effects in several animal model systems when compared to free drug. For example an antibody conjugate of a derivative of the vinca alkaloid, vinblastin, caused tumour regression in established lung and colon adenocarcinoma xenografts in nude mice whilst free drug at the same dose had only a slight effect (18). In clinical trials in patients with lung and colorectal cancer this vinblastin conjugate did not result in a significant clinical response (19). However, the dose that could be administered to patients was limited by severe duodenal toxicity. The monoclonal antibody used in this trial was directed against the KS1/4 tumour-associated antigen which, although having the advantage of being homogeneously expressed in high levels on colon carcinoma cells, is also expressed on a wide range of normal tissues including the duodenum. Thus, it seems likely that the side-effects seen in this trial were due to the lack of specificity of the monoclonal antibody. To date, in other clinical trials employing antibody-drug conjugates, there has been little evidence of significant clinical response and it thus remains to be demonstrated that sufficient cytotoxic drug can be delivered by monoclonal antibodies to human tumours to result in a significant therapeutic effect.

In an attempt to reduce the number of drug molecules that need to be delivered to a tumour more potent cytotoxic drugs are being explored which in their free form have little clinical utility due to severe adverse toxicity. An example of such conjugates are those prepared with trichosanthins. These are potent low molecular weight protein synthesis inhibitors which can kill cells at some 100–1 000-cell fold lower concentrations than most conventional anti-neoplastic cytotoxic drugs and thus less drug has to be delivered by the antibody per cell to result in cell death. In animal tumour models these trichosanthin conjugates have resulted in significant anti-tumour effects but to date clinical trials with such conjugates have not been reported (20).

Immunotoxins

Immunotoxins consist of monoclonal antibodies conjugated to potent toxins of bacterial or plant origin (4, 7). They are some 1 000–10 000-fold more potent than conjugates employing conventional anti-neoplastic cytotoxic drugs. Those most widely used in this approach are the bacterial toxins Diphtheria toxin and Pseudomonas exotoxin A and ricin from the seeds of the castor bean Ricinus communis. All three toxins contain binding sites which enable binding to the surface of most human cells. Once bound, the toxin is internalised, enters the cell cytosol and catalytically inactivates protein synthesis. Diphtheria toxin and Pseudomonas exotoxin A modify elongation factor 2 whereas ricin cleaves 28S RNA in the 60s ribosomal subunit. Since the toxins act catalytically only a small number of molecules need to gain access to the cell cytosol to kill.

Conjugates prepared with the intact toxins suffer from the drawback that they have non-specific toxicity associated with the binding activity of the toxin molecule. Modification of the binding sites has been achieved either

chemically or more recently by modification of the genes encoding the toxin and expression of mutated forms of the toxin lacking binding but retaining enzymic activity (21).

A widely used alternative strategy has been to use the isolated enzymic portion of ricin. Unlike the bacterial toxins in which the enzymic and binding domains reside in a continuous polypeptide chain, the enzymic domain of ricin (A-chain) is separated from the binding domain (B-chain) by a single disulphide bond. Cleavage of this bond liberates the A-chain from the B-chain. The A-chain is some 10 000-fold less toxic to animals than intact ricin since it contains no receptors for binding to cells. Immunotoxins prepared with ricin A-chain are extremely specific, the binding specificity being governed by the specificity of the monoclonal antibody. They can also be as potent as their intact toxin counterparts. However, a drawback of ricin A-chain immunotoxins is that only about one in five cell membrane reactive antibodies make potent A-chain immunotoxins (22). This is believed to be due to the fact that the B-chain of ricin is not only responsible for binding of the A-chain to the cell but also assists in cell entry. Certain antibodies can replace this entry function possibly by directing the A-chain to specific sites within the cell from which escape into the cytosol is possible. An alternative approach to using ricin A-chain is to utilise ribosome inactivating proteins (RIPs) such as gelonin, pokeweed antiviral protein and saporin (23). These RIPs are analogous to ricin A-chain, inhibiting protein synthesis by cleaving 28S rRNA and being relatively non-toxic since they lack a binding subunit.

Immunotoxins prepared with intact toxins generally use a non-cleavable linkage to attach the antibody to the binding subunit. Following internalisation of the conjugate into the cell the inhibitory subunit is released by reduction or peptide cleavage. In contrast, immunotoxins prepared with ricin A-chain or RIPs generally utilise a cleavable disulphide linker, because release of the free A-chain or RIP in the cytosol appears essential for the action of the immunotoxin. Novel hindered disulphide linkers have been developed for attachment of ricin A-chain to antibody. These minimise the breakdown of the immunotoxin in the bloodstream but allow release of the A-chain once the immunotoxin has been internalised. Unlike drug conjugates only a few (1–5) toxin molecules are attached per antibody.

Immunotoxins have been shown to inhibit tumour growth in a wide range of tumour models (4, 7). For example, an immunotoxin developed for the treatment of Hodgkin's disease gave complete remissions in mice bearing established Hodgkin's tumour xenografts (24). In the clinic, recent results with ricin A-chain and chemically blocked ricin immunotoxins directed against B-cell leukaemias and lymphomas have resulted in approximately a 50% response rate in phase 1 trials. Clinical trials employing immunotoxins to treat non-lymphoid cancers have been more limited and have produced less encouraging results. However, the conjugates used were far from optimal. For example, in a clinical trial with an anti-breast monoclonal antibody, 260F9, linked to *E. coli* expressed recombinant ricin A-chain there was some evidence of clinical response, but the immunotoxin had to be withdrawn after the treatment of only 9 patients due to debilitating neuropathies (25). The antibody used in this trial, although binding to a large percentage of breast tumours and forming a relatively potent immunotoxin, also bound to normal tissues including nerve Schwann cells. Again this is an example where the normal tissue binding of the antibody has limited the potential utility of an antibody conjugate.

Immunogenicity may be more of a problem with toxin conjugates since both the antibody and toxin are immunogenic. Indeed, in clinical trials with ricin A immunotoxins the antibody response to both components is seen approximately 10–12 days following the initial immunotoxin injection (26). With bacterial toxins this problem is likely to be more severe since a large percentage of the population are actively immunised against Diphtheria toxin or have been exposed to Pseudomonas exotoxin A as a result of infections. Humanisation of the antibody is unlikely to overcome the immunogenicity of the immunotoxin. However, both in animal and human studies, immunosupressive agents have been found to overcome this problem partially (27, 28).

In experimental systems the problems of antigenic heterogeneity limiting the clinical utility of immunotoxins has been addressed. Both cocktails of immunotoxins directed against different antigens (29) and combining an immunotoxin with cytotoxic drugs or cytokines (30), have been shown to produce superior anti-tumour effects than the use of a single immunotoxin.

Radionuclides

The radionuclides Iodine 131 and Yttrium 90 emit high and intermediate energy beta particles respectively and have half-lives of 2.7 and 8 days respectively which makes them suitable for tumour targeting (3, 8). The beta particles have path lengths that can span many cell diameters. Consequently, antibody conjugates prepared with these radionuclides do not need to be internalised to kill the cell. More importantly they can kill cells that surround the targeted cell allowing the destruction of adjacent tumour cells which either lack the target antigen or are in areas of poor vascularisation where the antibody conjugate cannot penetrate. Potentially, radionuclide conjugates can overcome both the problems of antigenic heterogeneity and tumour access. Unfortunately, since the beta particles can reach over many cell diameters these conjugates cause radiation damage to normal tissues while they are circulating in the bloodstream and this limits the amount that can

be administered. This problem is exacerbated for ^{131}I which emits both beta particles and high energy gamma emissions increasing the radiation dose to normal tissues. ^{131}I is generally attached to monoclonal antibodies by direct halogenation while ^{90}Y is attached via chelators which are chemically coupled to the antibody. Problems in vivo of dehalogenation in the case of ^{131}I and release of free radionuclide from the chelator in the case of ^{90}Y are being addressed by the development of more stable linker technology.

Monoclonal antibody conjugates of both radionuclides have been shown to cause regressions of tumours in animals in a number of studies (3, 8). As expected, dose limiting toxicity was to the bone marrow as a consequence of non-specific irradiation by circulating conjugate. There have been, or are in progress, over 30 clinical trials with antibody-radionuclide conjugates. Although complete and partial remissions have been reported in a proportion of the patients treated, these have been confined mainly to lymphoid cancers. As in the animal studies, bone marrow toxicity has limited the dose of conjugate that could be administered. One avenue of research which is being explored in an attempt to reduce this problem is the use of a two-stage system using a bispecific antibody in which one of the binding domains recognises the target antigen and the other domain recognises the chelated radionuclide. Following localisation of the unconjugated bispecific antibody at the tumour and clearance of the antibody from the bloodstream, the chelated radionuclide is administered. The bispecific antibody then traps the chelated radionuclide at the tumour site. Toxicity to normal tissues is minimised since the chelated radionuclide is rapidly cleared from the bloodstream. The technical feasibility of this approach has been demonstrated in tumour xenograft models using a chelate of the imaging isotope ^{111}In and an appropriate bispecific antibody (31). Radionuclides such as ^{212}Bi and ^{211}At which emit alpha particles have also been investigated as potential agents for targeting by monoclonal antibodies (32). Since the alpha particles traverse only one-to-two cell diameters non-specific toxicity is reduced. In experimental systems, conjugates of these radionuclides have been shown to be capable of killing tumour cells, but the short half-lives of these isotopes is likely to limit their clinical utility. An alternative strategy which has been explored is to attach the non-toxic, non-radioactive, isotope boron 10 to antibodies. Once targeted the ^{10}B is irradiated with low energy thermal neutrons which result in it undergoing nuclear fission to liberate alpha particles at the tumour site (33). This approah is limited since thermal neutrons do not penetrate tissue very well.

Enzymes

A new antibody-based targeting strategy that has recently been developed is antibody-directed enzyme prodrug therapy (ADEPT) (34). An enzyme capable of converting a non-toxic prodrug into a potent cytotoxic drug is covalently attached to a tumour selective monoclonal antibody. Following localisation of the antibody enzyme conjugate at the tumour site and clearance of residual conjugate from the bloodstream, the prodrug is administered which is converted by the enzyme into a potent cytotoxic drug at the tumour site, so minimizing non-specific toxicity. This approach has a number of potential advantages over the other targeting strategies. Tumour selective monoclonal antibodies which are not internalised and thus will not make potent drug conjugates or immunotoxins, can be used in this approach. Limitations in drug potency are overcome since a single enzyme attached to an antibody can generate a large number of cytotoxic drug molecules from prodrug molecules at the tumour site. Since the low molecular weight cytotoxic drug is generated outside the tumour cell it can diffuse rapidly to adjacent tumour cells which either lack the target antigen or are in areas of poor vascularisation. Thus ADEPT, like radionuclide targeting, can potentially overcome both the problems of antigenic heterogeneity and tumour access. Unlike radionuclide conjugates, however, both the antibody-enzyme conjugate and the prodrug should be relatively non-toxic and thus they should not result in toxicity when circulating in the bloodstream. However, non-specific toxicity will occur if residual antibody-enzyme conjugate remains in the blood-stream or normal tissues at the time of prodrug administration, if endogenous enzyme exists which can cleave the prodrug, if the antibody delivers the enzyme to normal tissues or if active drug generated at the tumour escapes back into the general circulation. Selection of an appropriate ADEPT system will have to aim to minimise these possibilities.

Enzyme and prodrug combinations used to date in this approach include carboxypeptidase G2 and a glutamyl derivative of a benzoic acid mustard alkylating agent (34), alkaline phosphatase and phosphate derivatives of etoposide or mitomycin C (35) and penicillin-V-amidase and a phenoxyacetamide derivative of doxorubicin (36). In each case the prodrug has been shown to be at least 50-fold less toxic than the free active drug in vitro against tumor cells and combinations of the enzyme conjugate and prodrug when administered to tumour bearing mice have given superior anti-tumour efficacy compared to free drug. For example, an anti-tumour monoclonal antibody-alkaline phosphatase conjugate in combination with etoposide phosphate caused approximately 40% complete remissions of established colon tumour xenografts whereas either the free drug etoposide or etoposide phosphate had very little effect on tumour growth (37). Specificity was demonstrated in this study since an alkaline phosphatase conjugate prepared with an antibody that did not bind to the tumour xenograft did not enhance the limited therapeutic effect of the etoposide phosphate. Most effort in the

ADEPT area is currently focused on identifying the optimal enzyme prodrug combination for clinical trials.

Conclusions

Monoclonal antibodies offer the opportunity for selective delivery of a range of anti-neoplastic agents to tumours. In animal model studies, significant antitumour effects have been demonstrated using monoclonal antibodies to selectively deliver anti-neoplastic drugs, toxins, radionuclides and enzyme prodrug combinations. Antibodies may also offer the opportunity of selectively delivering other agents of potential use in cancer therapy such as differentiation inducers (38) and radiosensitizers (39). It remains to be clearly established in the clinic if any of these approaches will have a significant impact in cancer therapy. However, encouraging results are emerging which suggest that both immunotoxins and radionuclide conjugates will have a role in the treatment of certain cancers.

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