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COMMENTARY

Novel Therapeutic Strategies to Selectively Kill Cancer Cells

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ABSTRACT. Tumor invasion, metastasis, and resistance to chemotherapeutic drugs or radiation are major obstacles for the successful treatment of cancer. To overcome some of these limitations, therapeutic strategies that increase the specificity and efficacy and reduce the toxicity of the anti-cancer drugs or toxins are being explored. Cancer cells overexpress specific protein antigens and carbohydrate structures that may function as cell surface receptors. These cancer cell specific markers can be exploited while designing new cancer therapies. Monoclonal antibodies that have been humanized to reduce immunogenicity and targeted to specific antigens on cancer cells, enzyme-monoclonal antibody/prodrug conjugates that will selectively kill the target cells following drug activation, and recombinant toxins are some of the novel classes of agents in development. Another novel approach being investigated to treat cancers is the use of inactive pore-forming toxins with built-in biological "triggers" that will activate the toxin following a biological stimulus. These pore-forming cytolytic toxins can be rendered active by tumor-specific proteases, that are often overexpressed in cancer cells, thereby targeting the toxic effects. Such pore-forming or membrane-acting toxins may serve as novel cytolytic agents against solid tumors, which, to date, have proved to be more resistant to conventional toxins. BIOCHEM PHARMACOL **55**;3:247–252, 1998. © 1998 Elsevier Science Inc.

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Several modalities including radiation, chemotherapy, and surgery, either alone or in combination, are used for the treatment of cancer. Despite their successes, there has been only modest improvement in the mortality rates for common cancers [1, 2]. A major drawback of radiation or chemotherapy is the often unacceptable damage to normal tissue at effective doses for eradicating cancer cells. Moreover, chemotherapeutic agents generally have not been effective in treating aggressive solid tumors because of poor penetration within the tumor mass [3, 4]. In addition, after surgical removal of solid tumors metastatic cells often remain, which can be resistant to conventional chemotherapy. Hence, new cancer therapies based on recent advances in biotechnology are being actively sought. A major consideration is to increase the therapeutic index by improving the specificity and efficacy and reducing the toxicity of the cytotoxic agents. One approach is to localize the cytotoxic agent by targeting tumor cells. To increase specificity and reduce toxicity, trigger mechanisms could be designed so that the toxic agents synthesized in their prodrug or inactive forms could be rendered active when and where required. Triggering signals could be either exogenous factors such as light or chemicals or endogenous cellular factors such as enzymes.

The unique features of cancer cells can be exploited in the development of targeting agents. For example, cancer cells often overexpress specific tumor antigens, carbohydrate structures, or growth factor receptors on their cell surface. Tumor-associated antigens such as the CD40 [5, 6], a Le^y-related carbohydrate antigen [7, 8], and the Erb-2 and epidermal growth factor (EGF) receptors [9, 10] are found to be highly expressed in a wide range of human malignant tumor cells. mAb† to tumor-associated antigens have been used to target cancer cells and deliver enzymes, low molecular weight drugs, and toxic proteins to these cells. In addition to tumor-specific antigens, some tumor cells also express unique proteases.

Tumor-associated proteases play an important role in several pathological conditions including the invasion and metastasis of cancer cells [11–14]. To date, these proteases have not been exploited effectively for therapy when compared with tumor-specific antigens. Many matrix metalloproteases and other degradative proteases are secreted as inactive or latent enzymes. However, under pathological conditions (such as malignancy), these enzymes are activated, or several of the tumor proteases are overexpressed [15, 16]. Certain proteases are also secreted by the host cells and contribute to the overall proteolytic activity of the

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† *Abbreviations:* mAb, monoclonal antibodies; V, variable region; V_H, heavy chain variable region; ScFv, single chain Fv fragment(s); αHL, α-hemolysin (α-toxin) of *Staphylococcus aureus*; and PE, *Pseudomonas* exotoxin A.

tumor [14, 17]. One novel strategy is to use these tumor-associated proteases for activating drugs or toxins to improve their specificity. In addition to their cancer association, some proteases are thought to play a causative role in other diseases such as rheumatoid arthritis [18] and Alzheimer's disease [19], thus suggesting that this approach could be used to treat diseases other than cancer.

STRATEGIES TO DELIVER TOXINS AND DRUGS TO TARGET CELLS

Based on the above concepts, various strategies for targeting cytotoxic agents are in development, including the following: (1) Antibodies against tumor-associated antigens or growth factors have been used to target the delivery of cytotoxic drugs, radionuclides, and bacterial toxins to the tumor cells. In the case of drug–mAb conjugates, the entire conjugate may be internalized and the drug released intracellularly, or the drug may be cleaved extracellularly and the released drug taken up by diffusion or active transport. Success with drug–mAb conjugates has been somewhat limited thus far because sometimes the drug requires intra- or extracellular chemicals or enzymes to be cleaved from the antibody. Furthermore, the drug delivery is limited by the number of drug molecules that are efficiently carried by each antibody. Radionuclides can also be linked to mAb. An anti-Tac mAb armed with ^{90}Y is currently being tested on patients with adult T-cell leukemia [20]. Another approach is to conjugate toxins to monoclonal antibodies. These conjugates are termed "immunotoxins," and several are in early clinical trials. (2) Prodrugs in conjunction with enzyme–mAb conjugates can also be used to target tumor cells [21–23]. Some prodrugs are inactive drug precursors that are not readily taken up by cells and hence are less toxic to the normal cells. The harmless prodrugs can be converted into active drugs by specific enzymes (e.g. alkaline phosphatase) that are covalently linked to an mAb that recognizes tumor-specific antigens. The active drug will then penetrate the nearby tumor cells, resulting in cell death. A number of such prodrug/mAb–enzyme conjugates have been developed and tested *in vitro* on cell lines [24] and for their *in vivo* anti-tumor activities in animal models [25, 26]. A significant advantage to this approach is that the complex does not require internalization, and a large amount of the drug can be generated at the tumor site. (3) Synthetic copolymers are also being developed as drug carriers. Drugs can be linked or combined with copolymers and activated by enzymes or light. Such combination agents are effective against tumors in animal studies [27]. (4) Liposomes are also effective as carriers for drug delivery [28–30]. A major advantage of this system is that liposomal components are nontoxic, nonimmunogenic, and biodegradable. However, their utility is limited because of the rapid uptake by phagocytic cells of the immune system. To obtain optimum drug delivery, the liposomes should be preferentially taken up by target cells, the drug should be quantitatively retained until delivery to target cells, and the clearance rate of the liposomes from the circulatory system should be under some control. To help overcome some of these limitations, a new class of liposomes called "stealth" liposomes has been developed [28]. The lipid bilayer of these liposomes contains glycolipids or lipids conjugated with ethylene glycol. Such liposomes have more stability, are not readily removed by the immune system, and may prove more useful in cancer chemotherapy. Other liposomes covalently attached to an mAb that recognizes specific T-cell surface antigens have been used to deliver the phototoxic drug pyrene, resulting in selective killing of T lymphocytes following irradiation [31]. Thus, by introducing such biological (enzyme) or physical (light) triggers into therapeutic agents, one may be able to optimize control and local delivery of the cytotoxic agents.

MONOCLONAL ANTIBODIES FOR DELIVERY OF THERAPEUTIC DRUGS OR TOXINS

Monoclonal antibodies are relatively large molecules (~150 kDa) and hence diffuse slowly and are not optimal targeting agents for the treatment of most solid tumors [4, 5]. With advances in protein engineering, efforts are aimed at reducing the size of the mAb. Recombinant ScFv fragments that are much smaller in size (~25 kDa) and only consist of the heavy and light chain variable domains connected by peptide linkers or disulfide bridges have been designed [32–34]. To further reduce the size of the antigen binding site, single V_H domains that are half the size of ScFv but retain most of the original antigen affinity in the absence of a light chain partner are being used [35, 36]. Such antigen binding V_H domains devoid of light chains were first detected in camel IgG [36]. Single human V_H domains were then designed by mimicking the camelid heavy chain sequences [37]. To develop single domain antibodies with increased specificity and affinity, antibodies have been reengineered using powerful techniques such as phage display. Recently, camelized human V_H antibodies that have increased folding stability have been obtained from phage-display libraries [38].

Another approach is using bispecific antibodies that combine the specificity of two different antibodies within one molecule and thus can cross-link an effector or killer cell with the target cell to be destroyed [39]. These agents are being developed for the therapy of cancer and infectious diseases. For example, a bispecific single chain antibody that can bind both the EGF and ErbB-2 receptors and linked to pseudomonas exotoxin A was developed recently, and its efficacy demonstrated *in vitro* and in an animal model [40].

Most mAb used as immunoconjugates are murine in origin. Hence, another barrier with immunotherapy is the development of an immunogenic response. This problem can be alleviated either by using immunosuppressive drugs (e.g. cyclosporin A), by derivatizing the mAb with polyethylene glycol [41], or by humanizing the antibody. Humanized antibodies have been constructed that retain the

high affinity binding from the mouse antibody determining region in the human V framework. These antibodies have the therapeutic advantages of a human antibody and have reduced immunogenicity and longer half-life [42–44].

SELECTIVE KILLING OF TARGET CELLS USING RECOMBINANT TOXINS

Based on their sites of action, toxins have been broadly classified into two groups: (1) Toxins that act within the cell, which must be internalized to produce their cytotoxic effect. Examples of toxins of plant origin are ricin, saponin, and gelonin, whereas the bacterial toxins include diphtheria toxin, pseudomonas exotoxin, and anthrax toxin. The plant toxins act by inactivating the eukaryotic ribosomal machinery, whereas the potent activity of the bacterial toxins is through ADP ribosylation of elongation factor-2, thereby inhibiting protein synthesis [45]. Some of these toxins have been conjugated with monoclonal antibody to produce immunotoxins that may serve as potential anticancer agents. (2) Membrane-acting toxins produce their cytolytic action by damaging the membrane or forming pores on the cell surface, ultimately resulting in cell death. A number of bacteria produce pore-forming toxins including staphylococcal α-hemolysin (αHL), streptolysin O, *Escherichia coli* α-hemolysin [see reviews in Refs. 46 and 47], pneumolysin [48], and aerolysin [49]. To name a few others, perforins are toxins produced by cytolytic lymphocytes [50], and equinatoxins are cytolysins produced by sea anemone [51].

Although there is a growing list of these toxins, our understanding of the structure, function, and mechanism of assembly is incomplete. The pore-forming property has been exploited for basic biological studies while reengineering these toxins for their potential application in biotechnology. An immediate application of such pore-forming toxins is to study intracellular processes by the controlled permeabilization of whole cells [52, 53] or certain regions of cells such as axons or cell bodies of neuronal cells [54]. Pores produced by different toxins range in size from 2 nm (α-hemolysin) to 30 nm (streptolysin O). Thus, by exploiting the different molecular selectivity of these pores, one can obtain controlled trafficking of molecules ranging in size from nucleotides to antibodies. More recent studies focus on reengineering the toxins for their potential application in biotherapeutics.

IMMUNOTHERAPY USING IMMUNOTOXINS

Immunotoxins are chimeric proteins consisting of a toxin coupled to an antibody by either chemical conjugation or genetic engineering. Growth factors or hormones that recognize specific receptors on the target cell have also been used in place of the antibody. Many antibody–toxin conjugates have been developed and tested for their efficacy *in vitro* and *in vivo* [55–57]. Besides the use of mAb, the specificity of these conjugates has been further enhanced by removal of the toxins' own receptor binding domain. Some of these immunotoxins are effective against hematologic malignancies and are in clinical trials [58, 59]. In a recent study, an immunotoxin LMB-1 was made by chemically linking B3 mAb to PE38, a genetically engineered form of PE, and was found to be effective in patients with advanced solid tumors [60]. Some of the problems encountered with these antibody approaches include the heterogeneity of tumor-associated antigen expression, poor penetration of the conjugate within tumor masses, and toxicity to normal cells [61]. Another limitation is that the toxin moieties of the present immunotoxins require internalization and translocation to the cytoplasm to achieve their cytotoxic effects. Therefore, the process of endocytosis has to be effective. To overcome some of these limitations, new approaches for immunotoxins must be designed.

MEMBRANE-ACTING IMMUNOTOXINS

The limiting clinical success using conventional immunotoxins made it important to explore other classes of toxins to create effective immunotoxins. Recently, attempts have been made by several research groups to use membrane-acting toxins as potential agents for killing target cells. For example, a membrane-acting hemolytic toxin from the sea anemone *Stoichactis helianthus* was linked to an mAb directed against carcinoembryonic antigen [62] or to an antibody that recognizes specific antigens expressed on immature T lymphocytes [63]. The phospholipase activity of the hemolytic toxin disrupts the cell membrane resulting in cell death. Another immunotoxin in development is phospholipase C (PLC), the α-toxin produced by *Clostridium perfringens*, that hydrolyzes the phospholipid phosphatidylcholine and is conjugated to the fab domain of the anti-Tac antibody [64].

PORE-FORMING TOXINS AS COMPONENTS OF IMMUNOTOXINS

To date, the potential use of pore-forming toxins to generate a novel class of immunotoxins has not been extensively explored. A new agent in testing is a conjugate of transferrin (a regulator of cellular growth and a potent mitogen) and equinatoxin II, a cytolysin from a sea anemone that is active against tumor cells *in vitro* [65]. Recently, the CytA-δ-endotoxin from the bacterium *Bacillus thuringiensis* conjugated to anti-Thy 1 monoclonal antibodies and insulin was developed and tested for its cytolytic effects *in vitro* [66].

Another pore-forming toxin is αHL, a water-soluble toxin secreted by *S. aureus* [67]. αHL is currently being explored to develop a novel class of immunotoxins called "proimmunolysins" (Fig. 1). Inactive mutants of αHL with a built-in biological trigger were engineered so that its activity could be switched on by specific proteases [68–70]. The use of tumor-protease-activated triggers to produce pore-forming activity at the surface of rabbit red blood cells

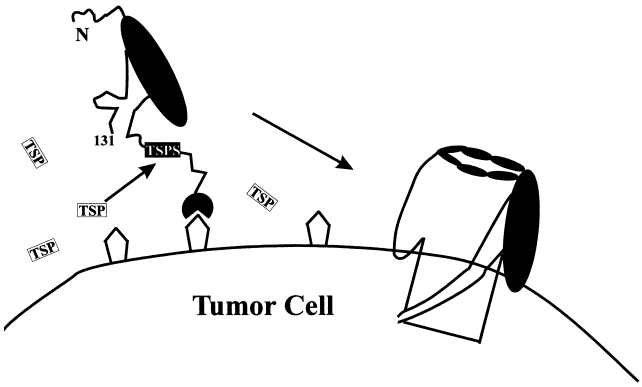

FIG. 1. Design of proimmunolysin. Inactive αHL prolysin is coupled to a monoclonal antibody that recognizes specific antigens on the target cell. Tumor specific proteases (TSP) secreted by the tumor cell will cleave at the tumor specific protease site (TSPS) in the redundant region of the overlap fragment of αHL. The resultant nicked form of the protein will adhere to target tumor cells and then kill them by damaging the membranes of the cells or increasing the permeability of the cells to other cytotoxic agents.

has been demonstrated recently [71]. Mutants of αHL that are rapidly and preferentially activated by tumor proteases might have important applications for killing cancer cells that secrete these proteases. To target the inactive αHL prolysin to tumor cells, the toxin would be coupled to a monoclonal antibody that recognizes specific tumor antigens on the target cell. The proimmunolysin would act on the cell surface and hence have an advantage over the conventional immunotoxins that require internalization. Activation by tumor proteases will add another degree of specificity beyond that conferred by the antibody as proimmunolysins attached to irrelevant cells would remain inactive. It differs from pore-forming immunotoxins, in that a triggering signal has been engineered to target activation of the toxin. Thus, the conjugate by itself should be noncytotoxic to the normal cells. Design of such a therapeutic agent takes advantage of two separate properties exhibited by cancer cells (expression of tumor-associated antigens and overexpression of proteases), thereby improving the therapeutic index. Immunotoxins made with such pore-forming toxins may be more useful for treating solid tumors since they do not have to overcome the problem of penetrating the tumor mass. Alternatively, pore-forming toxins that form large pores might be used to increase the efficacy of the conventional immunotoxins by allowing their easy access into the cytosol.

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