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Review Article

Chimeric proteins as candidates for cancer treatment

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CONTENTS

Abstract

The rising rate of cancer-related diseases and mortality underscores the need for new approaches to directly target and fight cancer. This review summarizes one of these new promising treatments, the use of targeted chimeric proteins. Chimeric proteins are two proteins fused at the DNA level in such a way that, once expressed, they result in a single polypeptide chain consisting of two moieties: a targeting moiety (usually a cytokine or growth factor) and a killing moiety (usually a bacterial or plant toxin). Many chimeric proteins have been constructed and developed over the years for the treatment of a variety of malignancies and these molecules are the main scope of this review. Moreover, this review presents new approaches for battling cancer, such as recruiting the apoptotic machinery via chimeric proteins, the use of receptor-mediated delivery of toxin-DNA, or T-cells as vehicles for delivering immunotoxins, all of which are trying to develop specific, efficient, nontoxic and non-immunogenic reagents for targeted cancer treatment.

Introduction

It has been estimated that over 1.2 million Americans will be diagnosed with cancer in the year 2002, and about half of them will die (1). To date, treatment of malignancies consists of radiation, chemotherapy, surgery or a combination of these procedures. Since these treatments are not specific to cancer cells, they cause many side effects. Moreover, there is always the risk that some of the cancer cells will not be eliminated by these treatments. Therefore, there is a great need for new approaches to fight cancer. This review summarizes the use of molecules termed chimeric proteins as potent reagents for targeted cancer therapy.

First generation molecules: natural toxins

One of the major goals in medicine today is the design of therapeutic agents that can selectively kill and eliminate certain populations of cells in the human body without harming neighboring healthy cells. This concept was in fact conceived by Paul Ehrlich who over a century ago suggested that drugs could be bound to specific carrier vehicles that would deliver them specifically to their site of action. These carriers were designated “magic bullets”, which, theoretically, could cure all diseases, providing their pathologies were known (2, 3). An important aid in achieving efficient targeted therapy is to identify specific surface markers, mainly receptors/binding sites, overexpressed on malignant cells as compared with their normal counterparts. Despite the variety of approaches used, only a limited number of tumor-associated markers have been described for each histological type of cancer.

The first tumor markers were detected by biochemical procedures. Subsequently, surface markers were determined by serological techniques. The first magic bullets used whole antibodies as carriers to deliver medications into cancer cells. These attempts often failed due to the polyclonality of the antibodies and because of the small amounts of antibodies that could be produced. The advent of the monoclonal antibody helped to overcome

both these limitations and they have been used for targeted therapy (4). This technology has led to the discovery of numerous cell surface molecules associated with cancer cells. Based on the differential expression patterns of these molecules, improved targeting molecules have been designed to specifically target and destroy cells expressing such surface molecules. Chemotherapeutic agents such as daunorubicin, methotrexate and vindesine, or a radioactive isotope have been chemically conjugated to monoclonal antibodies (Fig. 1a) and tested in clinical trials (5-7). A recent example is SGN-15, a monoclonal antibody-doxorubicin conjugate for the potential treatment of epithelial tumors (colon and breast cancer) which is currently in phase II trials (8, 9). Anti-CD33 conjugated to iodine-131 or bismuth-213 were constructed for the treatment of acute myelogenous leukemia (AML) and chronic myelogenous leukemia (CML) (10). An anti-CD20 conjugated to iodine-131, known as tositumomab, has shown promising results in the treatment of patients with lymphomas (11, 12).

Researchers became interested in substances more potent than chemotherapeutic agents such as toxins that act catalytically so that even a single molecule entering the cell could cause it to die. These molecules, termed immunotoxins, were designed by the chemical conjugation of whole monoclonal antibodies to bacterial or plant toxins (Fig. 1a). Mutating or deleting the ability of the toxin to bind to its own receptor partially eliminated the nonspecific toxicity associated with these natural bacterial or plant toxins (13-19).

In the search for proteins that could cause cell death, a number of protein families were suggested. The most widely used killing domains in immunotoxins are bacterial and plant toxins, including *Pseudomonas* exotoxin, diphtheria toxin, ricin A and many other toxins or derivatives of these molecules.

Pseudomonas *exotoxin*

One of the most widely used toxins in chimeric proteins is *Pseudomonas* exotoxin (PE) produced by the bacterium *Pseudomonas aeruginosa*. X-ray diffraction analysis has shown that PE is composed of 3 different domains. Functional analysis of deletion mutations in the PE gene revealed that domain I is responsible for cell recognition, domain II for translocation of the toxin across membranes and domain III for ADP-ribosylation of elongation factor 2. Having accessed the cytoplasm, PE inhibits protein synthesis by its ADP-ribosylation activity, thus causing cell death (20). The PE receptor has been identified as low-density lipoprotein receptor-related protein (LRP) (21) and its significance in the process of PE intoxification has been clearly established (22, 23). If the whole toxin is used, nonspecific toxicity occurs mainly due to binding of the toxin to cells via its binding domain. To eliminate the nonspecific binding, 4 point mutations were introduced into the binding domain, abolishing its binding ability (PE^{4glu}). An alternative approach was to remove the binding domain, which did not affect the enzymatic activity. This approach gave rise to the molecules PE40 and PE38 (13, 14).

Diphtheria toxin

Diphtheria toxin (DT) is secreted from *Corynebacterium diphtheria*. It is translated along with a 25 amino acid signal peptide and is cotranslationally secreted as a single 535 amino acid residue protein with a molecular weight of 58 kDa. The protein contains 2 disulfide bridges. When the X-ray crystal structure of DT was resolved, the boundaries between the 3 domains of DT were delineated catalytic, transmembrane and receptor binding domains. DT is targeted specifically to cells expressing the heparin-binding epidermal growth factor-like DT receptor. DT, like PE, causes cell death via inhibition of protein synthesis by ADP-ribosylation of elongation factor 2 (13, 14, 24). The DT derivatives used abolish the ability of DT to bind to its own receptor. These include DAB486 (a full-length DT with point mutations) and the truncated forms of DT-DAB389 and DT388 (13, 14).

Ricin

Ricin is a 60 kDa glycoprotein derived from the caster bean. After entering the cell, ricin modifies the 28s rRNA, thereby inactivating ribosomes and resulting in cell death. The 3-dimensional crystal structure shows that ricin consists of 2 chains (25): an A subunit responsible for the enzymatic activity of ricin and a B chain which is its binding domain. When used in immunotoxins, the ricin A subunit was fused to the targeting domain (2, 26).

Similar to ricin, there are other ribosome inactivating proteins (RIPs) that are used as killing domains. These include saporin, gelonin, pokeweed antiviral protein, *etc*. (2). In addition, a number of cellular proteins such as RNaseI (27) and phospholipase C (2) have been used as the killing domain in immunotoxins and chimeric proteins (see below).

Second generation molecules: recombinant immunotoxins

As described above, monoclonal antibodies conjugated to bacterial or plant toxins were constructed and tested in clinical trials. Examples are Ki-4.dgA, an anti-CD30 monoclonal antibody coupled to ricin for the treatment of Hodgkin's and non-Hodgkin's lymphoma (28), and IgG-RFB4-SMPT-dgA, used to target patients with B-cell lymphoma (29). The immunotoxin LMB-1 is composed of monoclonal antibody B3 chemically linked to PE38. B3 recognizes a carbohydrate antigen (Le(Y)) present on many human solid tumors. LMB-1 was used for the treatment of a variety of carcinomas (30-33).

The evolution of genetic engineering made it possible

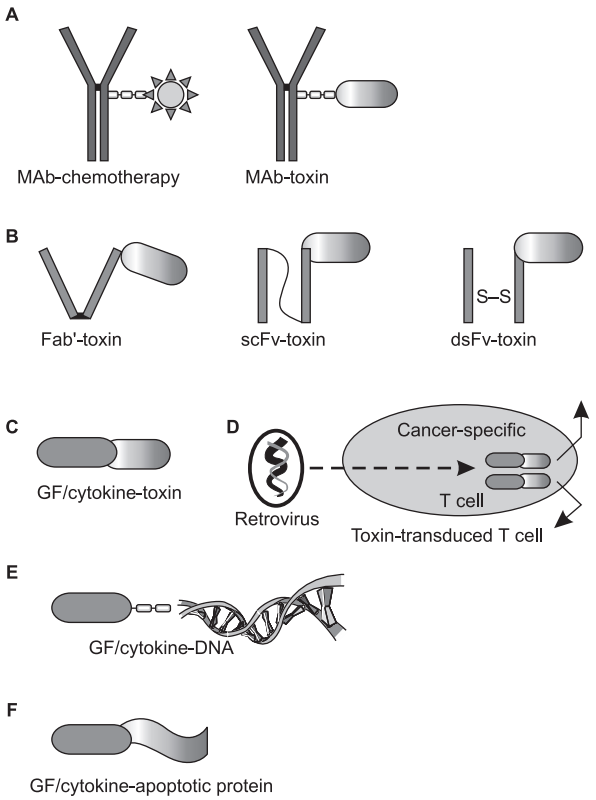

Fig. 1. Schematic representation of immunotoxins/chimeric proteins.

to genetically fuse 2 different proteins at the DNA level. The expressed fusion product is a single polypeptide with 2 moieties: a killing domain and a targeting domain. When monoclonal antibodies were used to treat human patients, they elicited an immune response against these antibodies. To overcome this difficulty, fragments of antibodies were used instead of the whole antibody to direct the toxin to the target cell. Such fragments are Fab, the single chain Fv (scFV) and disulfide bond Fv (dsFv) (34-36). A schematic representation of the different antibody fragments is presented in Figure 1b. Although all 3 recombinant immunotoxins exhibited a reduction in both nonspecific toxicity and immunogenicity, these problems were not eliminated. Examples of such molecules that have reached the clinical trial stage are Fab'-RFB4-dgA, an anti-CD22 Fab' fused to ricin for the treatment of B-cell lymphoma (37), anti-Tac(Fv)-PE40 for the treatment of leukemia (38), BL22, a scFv anti-CD22 fused to truncated PE for treatment of chemotherapy-resistant hairy cell leukemia (39), LMB-2, a single chain Fv anti-IL-2 receptor fused to the truncated PE (PE38) for the treatment of hematological malignancies (40) and the disulfide-stabilized recombinant immunotoxin RFB4(dsFv)-PE38 which acts on fresh malignant cells from patients with B-cell leukemias (41, 42).

Chimeric proteins

Although immunotoxins based on antibodies or fragments of antibodies as their targeting moiety acted as potent killing agents, they still caused nonspecific toxicity and, at sufficiently high concentrations, they damaged normal cells that did not express the specific target antigen. Researchers speculated that smaller molecules (*e.g.*, cytokines, growth factors [GFs], hormones, *etc.*) could achieve the desired specificity in targeting cancer cells. This concept arose from the notion that cancer cells have more GF receptors than normal cells and are usually involved in an autocrine, paracrine GF exertion loop allowing them to proliferate without control. In patients, several hundreds to thousands of these receptors are typically expressed per malignant cell. These include receptors for cytokines, interleukins, hormones, *etc.* Those molecules containing such small molecules as their targeting moiety were termed chimeric proteins (Fig. 1c). In the remaining section of this review we will present a number of examples for the use of chimeric proteins in cancer treatment.

Interleukins

1) Interleukin-2

Interleukin-2 (IL-2) was one of the first cytokines to be discovered and characterized (43). IL-2 is the main cytokine in the immune system and it has pleiotropic effects on T- and B-lymphocytes and other hematopoietic cells (44). The effects of IL-2 are mediated through a specific cell surface receptor (IL-2R) which is compromised of at least 3 subunits (44). In normal resting T- and B-cells, the expression of the high affinity IL-2R is minimal. In contrast, it is highly expressed in certain leukemias and lymphomas (45). Moreover, IL-2R is also highly expressed on activated T-cells in certain autoimmune diseases and as a result of graft rejection (45). Therefore, IL-2R is an attractive target for T-cell-directed therapy (24, 46).

A large number of IL-2-based chimeric proteins were constructed, among them the IL-2-PE chimeric proteins. We constructed and purified an IL-2-based chimeric protein termed IL-2-PE_{40} (47). Using this chimera, we were able to show that targeting of IL-2R-positive cells provides effective and selective immunosuppression. IL-2-PE_{40} delays and mitigates adjuvant-induced arthritis in rats (48), significantly prolongs the survival of vascularized heart allografts in mice (49), reduces the incidence and severity of experimental autoimmune uveoretinitis in rats (50), prevents the characteristic features of experimental autoimmune encephalomyelitis in rats (51) and mice (52), significantly reduces the clinical rejection score and cumulative rejection rate of orthotopic corneal grafts in rats (53) and suppresses the growth of a T-cell lym-

phoma in mice (54). All of these studies have been reviewed (55).

The first chimeric protein approved by the Food and Drug Administration for use in human patients was DAB389IL-2 (Ontak), which is indicated for the treatment of cutaneous T-cell lymphoma. The molecule, consisting of a truncated form of the bacterial protein DT fused to IL-2, proves that chimeric proteins can be useful pharmaceutical agents (18, 24). DAB389IL-2 is also used for treatment of other neoplasms. A phase II clinical trial to evaluate DAB389IL-2 for the treatment of chronic lymphocytic leukemia has recently been launched (24).

2) Interleukin-3

Interleukin-3 (IL-3) is a cytokine that supports the proliferation and survival of multipotential and committed myeloid and lymphoid progenitors (56). It has been reported that the IL-3 receptor (IL-3R) is absent from mature mononuclear cells and some primitive stem cells, and therefore, IL-3 does not act on most stem cells (57). CML progenitors have been found to express both IL-3 and its receptor, resulting in an autocrine loop that stimulates proliferation. In addition, binding of IL-3 to the IL-3R results in their rapid internalization. Based on these observations, 2 laboratories synthesized DT fusion proteins directed to the mouse IL-3R (58, 59). Later, Frankel *et al.* fused the human IL-3 to DT (DT388) for the treatment of patients with acute phase CML and AML, the most common types of acute leukemia in adults. Most AML patients receive chemotherapeutic drugs, although a large proportion of them are resistant to these drugs, resulting in frequent recurrent of the disease (57, 60, 61).

The DT-IL-3 chimeric protein caused the death of a number of AML blasts. In a study by Alexander *et al.*, it was shown that 92% of AML cell samples from patients displayed the high affinity IL-3R on their surface (60, 61). Unfortunately, these investigations suggest that there is considerable variability in the expression of the IL-3R among blasts isolated from a single patient, with only about 33% exhibiting sensitivity to the chimeric protein. Due to the variability in IL-3R expression on leukemic blasts, DAB389IL-3 is unlikely to provide any therapeutic benefit in patients suffering from myeloid leukemia (24, 60, 61).

3) Interleukin-4

Interleukin-4 (IL-4) is a pleiotropic immunoregulatory cytokine that upregulates adhesion molecules, inhibits cell proliferation and mediates signal transduction in tumor cells (62). IL-4 is produced by activated T-lymphocytes and mast cells. It inhibits the growth of several tumor cell lines and is known to induce apoptosis in human breast cancer cells (63). Since a wide variety of human carcinomas express the IL-4 receptor (IL-4R), IL-4 can be used to target chimeric proteins to these cells.

To target the IL-4R, Kawakami *et al.* fused a circular, permuted IL-4 to a truncated PE (PE38), thus producing the IL4(38-37)-PE38KDEL chimeric protein. This chimera, also termed cpIL4-PE, proved highly cytotoxic to IL-4R-positive cell lines (62, 63). A correlation was found between the expression of the IL-4R and the cytotoxicity of the chimera. cpIL4-PE was constructed for the treatment of a large number of cancers, all expressing the IL-4R. These include medulloblastoma (64), AIDS-related Kaposi's sarcoma, glioblastoma multiforme and breast cancer (62). Phase I clinical trials with cpIL4-PE for the treatment of glioblastoma multiforme showed promising results without detectable toxicity to normal brain tissues (62). Other clinical trials using the cpIL4-PE for the treatment of high-grade glioma showed antitumor activity when administered intratumorally (65). Testing of this chimeric protein as a possible treatment for other brain cancers is under way (64).

IL-4 was also fused to DT. This chimeric protein, termed DAB389IL-4, inhibited protein synthesis in AIDS-related Kaposi's sarcoma cells at very low concentrations (IC_{50} of 5×10^{-11} M) in *in vitro* experiments (66).

4) Interleukin-13

Interleukin-13 (IL-13) induces the humoral immune response by stimulating the proliferation and survival of activated B-cells. IL-13 has many indirect modes of action. These include mediating fibrosis in allergic asthma and inhibiting the Th1 response through its inhibition of IL-12 synthesis in monocytes and macrophages. The IL-13 receptor (IL-13R) appears to be overexpressed on human brain tumor cells and is considered to be a marker and a therapeutic target for human high-grade glioma (67, 68). In addition, IL-13 and its receptor are usually coexpressed in Hodgkin's lymphoma cells (69).

Glioblastoma multiforme, one of the most malignant central nervous system tumors of glial origin (gliomas), is characterized by the presence of necrosis, vascular proliferation and aggressive invasion of the surrounding normal brain tissues. Most gliomas, including glioblastoma multiforme are incurable, despite surgery, radiotherapy and chemotherapy (68). The IL13-PE38QQR was constructed to utilize the IL-13R as a therapeutic target. This chimeric protein is composed of human IL-13 fused to a mutated form of PE. For specific targeting of IL-13R in gliomas, the human IL-13 was mutated to alter IL-13 interaction with the shared functional IL-13/4 normal tissue receptor, but not with the glioma-associated receptor (70). This recombinant protein is highly cytotoxic to various IL-13R-positive solid tumor cells, including glioblastoma cell cultures and 2 xenograft models. Clinical trials are now being conducted with IL13-PE38QQR for the treatment of refractory glioblastoma multiforme (71).

Li *et al.* developed the DT390IL-13 chimeric protein for the treatment of glioblastoma multiforme. The chimera was able to inhibit the growth of a glioblastoma multiforme cell line. In a xenograft model, DT390IL-13 caused complete regression in most animals after 5 intratumoral

injections (72). Therefore, this chimera, like IL13-PE, may provide an alternative therapy for brain cancer.

Growth factors and hormones

1) Granulocyte macrophage colony-stimulating factor

Granulocyte macrophage colony-stimulating factor (GM-CSF) is a cytokine responsible for the growth, differentiation and functional enhancement of granulocytes and macrophages (73, 74). GM-CSF receptors are expressed on the majority of myeloid leukemias. However, these receptors are poorly expressed on early normal hematopoietic stem cells (75). Therefore, GM-CSF receptor is a good candidate for targeting by chimeric proteins.

DAB388-GM-CSF, or DTGM, is a fusion protein comprising residues 1-388 of DT fused to human GM-CSF. To test the potential therapeutic effect of DTGM in AML, an *in vivo* study was conducted using the SCID mouse model for acute myeloid leukemia. Administration of DTGM significantly prolonged host survival (76). Interestingly, in DTGM-treated mice that developed leukemia (22%), there was no apparent difference in the number of GM-CSF receptors on blasts, ligand affinity or sensitivity to DTGM, as compared to controls. It remains to be determined if this effect is due to the incomplete penetration of the drug into tissues or to some unknown mechanism of cellular resistance against DTGM (24).

Based upon the initial success with this chimera, a series of preclinical studies were conducted in both mice and monkeys (77). Given the selectivity of DTGM for malignant or differentiated myeloid cells, with little toxicity to myeloid progenitors or other organs, phase I clinical trials are being carried out to test the potential use of DTGM as a therapeutic agent in AML.

2) Transferrin

The transferrins are a family of iron binding proteins. They are usually monomers with a molecular weight of about 80 kDa that control the level of free iron in body fluids (78). Transferrins and the transferrin receptor complex are well characterized. Therefore, this system has been exploited for the delivery of chimeric proteins to malignant cells overexpressing the transferrin receptor (79).

Tf-CRM107 was constructed for the treatment of malignant brain tumors. Tf-CRM107 is composed of transferrin fused to DT lacking receptor binding activity. This 140 KDa chimeric protein was able to inhibit tumor growth in animal models and in patients with malignant gliomas when injected intratumorally (80).

Tf-CRM107 at high doses can cause a neurological deficit consistent with endothelial damage. A recent study by Hagihara *et al.* (79) was conducted to overcome this drawback. In their study, animals were treated with Tf-CRM107 combined with chloroquine, a drug used to treat malaria. The drug accumulates in lysosomes and neutralizes vesicular pH. This combination treatment which allows the antitumor effect of Tf-CRM107 with suppressed nonspecific toxicity, shows great promise for the treatment of malignant gliomas.

In addition to DT, transferrin has been fused to ricin A (Tfr-RTA) (81, 82) and PE (83). Tfr-RTA is being evaluated for the treatment of leptomeningeal carcinomatosis (82). In contrast to Tfr-RTA, there has been little development of the transferrin-PE chimera.

3) Gonadotropin-releasing hormone

Gonadotropin-releasing hormone (GnRH) is a decapeptide normally synthesized by hypothalamic neurons and secreted into the hypophysioportal circulation via the portal vessels. GnRH is synthesized as a large peptide that matures through proteolytic processing and amidation at its C-terminal glycine. Upon reaching the anterior pituitary gland, GnRH selectively stimulates the gonadotroph cells to release luteinizing hormone and follicle-stimulating hormone, thus playing a central role in the neuroendocrine control of human reproduction.

The involvement of GnRH has been demonstrated in several carcinomas (84-86). GnRH-specific binding sites have been reported in some solid tumors as well as in established cell lines, although the functional role of these binding sites in human neoplasms remains obscure. Numerous analogs of GnRH have been developed, mainly to overcome its extremely short half-life and to enhance its affinity for the GnRH receptor. Recent data showing a response to GnRH analogs of nongynecological tumors, *e.g.*, cancers of the pancreas and liver, led us to explore the possibility that expression of the GnRH receptor/binding site may be a common phenomenon among neoplasmic diseases and, thus, an attractive target for selective therapy (84-86).

A number of GnRH-based chimeric proteins were constructed with an analog of the decapeptide GnRH (GnRH-trp^6 in which tryptophan replaced glycine in the 6th position) serving as the targeting domain, fused to the full-length mutated PE moiety of 66 kDa or to the truncated form of PE (PE40). Strong growth inhibition and killing of a surprisingly wide variety of cancers was found in both cancer cells arising from hormone-responsive tissues, as well as from nonresponsive ones, such as ovarian, breast, endometrial, cervical, colon, lung, hepatic and renal carcinomas, all confined to the adenocarcinoma type. This cytotoxicity is specific as it was blocked by the addition of excess GnRH. The specificity of the GnRH-PE66 chimeric toxin was also confirmed by GnRH binding assays (84).

When a polylinker was introduced between GnRH and PE to separate the killing domain from the target domain, enabling the molecule to fold properly, greater potency of the molecule was observed. The new chimera termed L-GnRH-PE$_{66}$ (87) was able to inhibit tumor growth in colon carcinoma xenografted nude mice. In

view of its specificity, L-GnRH-PE_{66} appears to be a promising candidate for the treatment of adenocarcinomas (85, 86).

4) Miscellaneous

A large number of additional cytokines and growth factors have been used in chimeric proteins that were constructed for the treatment of a variety of malignancies. These include mainly IL-6, TGF-α, fibroblast growth factor and epidermal growth factor (2, 13). However, further development of these reagents for clinical use has not progressed in recent years.

Disadvantages of bacterial-based chimeric proteins

Although several chimeric proteins are already in clinical use, they have several disadvantages that limit their clinical application. Since every chimeric protein displays some degree of nonspecific toxicity, at sufficiently high concentrations they damage normal cells that do not express the specific target antigen. These mainly affect hepatocytes, as reflected by abnormal liver function tests, or the vascular endothelium resulting in vascular leak syndrome (18, 88). The nonspecific liver toxicity of PE-based immunotoxins is probably due to easy access and very rapid nonspecific uptake and internalization of proteins by the hepatocytes. The normal tissue mediating these injuries, liver or vascular endothelium, could be the one showing the observed toxicity or the damage could be secondary, *i.e.*, macrophages taking up these molecules could be stimulated to release cytokines, causing injury to the liver and blood vessels.

Recently, experiments using an *in vivo* model consisting of a human neonatal foreskin xenograft in SCID (immunodeficient) mice helped to identify a 3-amino acid motif present in protein toxins causing vascular leak syndrome (89). Thus, in the future vascular leak syndrome induced by immunotoxins/chimeric proteins could be prevented by the use of antiinflammatory agents to block cytokine action and by the use of mutations or peptide inhibitors to prevent binding to endothelial cells.

Another major drawback in the clinical application of these molecules is the human immune response they elicit, mainly toward the toxin moiety. Bacterial toxins such as PE and DT are highly immunogenic and cannot be humanized by standard techniques. A prominent example is DT-derived immunotoxins, as most people in developed countries have been vaccinated against DT and many adults have neutralizing antibodies to DT. Immunogenicity is a problem to which, so far, no practical solution has been found. Once this hurdle is cleared, the concept of targeted therapy by chimeric proteins would be greatly advanced. Another major limitation in targeted therapy by immunotoxins/chimeric proteins is the fact that only a small proportion of the injected dose reaches the cancer target, generally less than 0.001% (90). Thus, it is evident that new approaches are needed to produce novel, improved targeting molecules, mainly composed of human, nonimmunogenic toxins.

Novel approaches

T-cells as vehicles for delivering immunotoxins

At the present time, there are new approaches for cancer treatment based on targeted therapy by chimeric proteins/immunotoxins. One such new strategy exploits the potential benefit of chimeric proteins/immunotoxins by combining targeted therapy with gene therapy. As mentioned previously, one major limitation in the use of immunotoxins for cancer treatment is the fact that only a small percentage of the injected dose ever reaches the cancer target. Thus, numerous laboratories have been exploring the application of adoptive immunotherapy with cancer-specific T-cells which can access *in vivo* tumors in a way no biological agent can (Fig. 1d). Chen *et al.* were the first to address this issue with a new class of tumor-specific killer cells. These cells are genetically modified lymphocytes that produce and secrete an svFV immunotoxin based on PE and recognizing Her2/Neu expressing human breast cancer cells (91). The transduced lymphokine-activated killer cells were shown to be strongly and selectively cytotoxic against tumors in culture and in nude mouse models. Using this approach, for the first time it was proven that mammalian cells can be genetically modified to produce and secrete targeted toxin proteins while remaining viable. This is most probably due to the remarkable conserved separation between the intracellular endoplasmic reticulum lumen and the cytosolic compartments, so that even minute amounts of toxin are prevented from gaining access to the ribosomes or cytoplasm. Similarly, Vallera *et al.* suggested the use of antigen-specific cytotoxic T-cells as vehicles for delivering immunotoxins to the cancer cell site in order to overcome this shortcoming. A retroviral vector was constructed for gene therapy with a DT-based immunotoxin. According to one report, IL-4 was fused to DT to target IL-4R on acute myeloid leukemia cells in a mouse model (92).

In another case, DT was fused to vascular endothelial growth factor (VEGF) to target the VEGF receptor on endothelial cells in the tumor neovasculature (93). The retroviral vectors were transiently transduced into $CD8^+$ cytotoxic T-cells specifically recognizing a precise antigen. Transduced cells expressing the fusion toxin, as opposed to the nontranduced control, were shown to significantly inhibit the growth of a s.c. tumor. Vallera *et al.* also showed that in the treated mice there was no sign of the renal or hepatic toxicity common to this class of immunotoxins, indicating that a retroviral-immunotoxin strategy allows more direct delivery to the site of interest. This approach takes advantage of a self-delivery system, but its major limitation is that self cytotoxic T-cells, which recognize a specific antigen, have to be tailored for each patient.

Receptor-mediated delivery of toxin-DNA

Another tactic for overcoming the immunogenicity associated with bacterial or plant-based immunotoxin/chimeric proteins is the targeted delivery of DNA encoding a toxin via selective receptors instead of targeting the protein-toxin itself. Such ligand-mediated endocytosis can specifically deliver DNA to cells bearing the appropriate cognate receptors (Fig. 1e). In a study by Hoganson *et al.* (94), the ligand basic fibroblast growth factor (FGF2) was used to deliver therapeutic genes to cells bearing the high-affinity FGF receptors. The DNA must encode proteins with high intrinsic activities, such as saporins (SAP, a ribosomal inactivating protein), so that even low expression of this DNA will kill the target cell. The main difficulty encountered is that these DNA-toxins can inhibit their own translation, thus compromising their efficacy. However, it was shown that FGF2-mediated delivery of saporin DNA resulted in a 60% decrease in cell number (94). Similarly, Kleeff *et al.* (95) demonstrated the targeted delivery of a suicide gene via the same FGF receptors in pancreatic cancer cells. Nevertheless, in this approach, the ability to deliver the toxin's DNA to the cells is controlled by a gate at the cell surface, by trafficking of the DNA to the nucleus and finally by transcriptional and translational control points. Moreover, the stability between protein-DNA fusions must also be addressed to ensure the success of this approach.

Apoptosis-inducing chimeric proteins

In our laboratory, we are now developing a new generation of chimeric proteins composed mainly of human, nonimmunogenic toxins. We took advantage of one of the most crucial proteins controlling body homeostasis, apoptosis-inducing proteins. These proteins are of human origin and they have a strong apoptotic effect when overexpressed. Moreover, they are downregulated in several malignancies and thus their reintroduction potentiates cell death. Our strategy is based on the delivery of less immunogenic human apoptotic proteins as novel killing components, instead of the bacterial or plant toxins (Fig. 1f).

Apoptosis, or programmed cell death, a mechanism intrinsic to all cells in the human body, plays an important role in development and homeostasis (96, 97). Disturbances in the regulation of apoptosis illustrate its importance in normal homeostasis (98). Abnormal resistance to the induction of apoptosis correlates with malformations, autoimmune diseases and cancer. In contrast, enhanced apoptotic decay of cells occurs in acute pathologies (infection by toxin-producing microorganisms) as well as in chronic diseases such as AIDS. Initially identified in *Caenorhabditis elegans* (99), 3 protein families are involved in promoting or inhibiting apoptosis: the Bcl-2 family, Apaf-1 and the caspases. Each family includes a large number of apoptotic proteins that can serve as the killing moiety of a chimeric protein (96, 97).

To validate the use of proapoptotic proteins as the killing moiety, we chose a well-known target, the IL-2R, in constructing the first chimeric protein, IL2-Bax (100). Here, the killing domain is Bax, a proapoptotic protein of the Bcl-2 family. Bax is a central protein in the apoptotic cascade and was shown to promote cell death via different pathways. The first prototype molecule, IL2-Bax, specifically targets IL-2R-expressing cells and induces cell-specific apoptosis (100).

Moreover, we showed that the IL2-Bax chimeric protein causes cell death by apoptosis via a mechanism similar to the natural response to a physiological stimulus such as serum withdrawal. The activity of IL2-Bax was evident in primary cultures taken from human donors (100) as well as in bleomycin-sensitive mice which are a model for idiopathic pulmonary fibrosis, also referred to as cryptogenic fibrosis alveolitis, a progressive interstitial lung disease of unknown etiology (101, 102, our unpublished results).

Next, we constructed a number of GnRH-based chimeric proteins fused to the proapoptotic proteins Bax, Bik and Bak of the Bcl-2 family (103) and to other proapoptotic proteins (our unpublished results). These chimeric proteins specifically target and kill adenocarcinoma cells. In addition, preliminary results show that in a colon carcinoma xenograft model in mice, one such chimeric protein is able to inhibit tumor growth (our unpublished results). Since these various chimeras induce cell-specific apoptosis both *in vitro* and *in vivo*, our novel approach could be applied to a wide variety of adenocarcinoma cells.

Our approach of utilizing human self proteins has several advantages. First, targeted cells can be selected and then eliminated through apoptosis, the natural mode of cell death. Killing target cells via the apoptotic pathway minimizes any tissue damage or systemic response. The apoptotic cells shrink and condense, while the organelles and plasma membranes retain their integrity. The dead cells are then rapidly phagocytized by the adjacent cells or macrophages, disappearing before any leakage of their content. Second, proapoptotic proteins are intracellular proteins of human origin and, as such, they are expected to display reduced immunogenicity in human recipients, thus overcoming the major obstacle involved in the use of bacterial and plant-based chimeric proteins. Third, these apoptosis inducing protein-based chimeras are sufficient to induce apoptosis in the target cells in the absence of any additional death stimuli. Despite the fact that these chimeric proteins are delivered from the exterior of the cell, we showed that they are allocated to the same compartments as their endogenous counterpart proteins and induce apoptosis in a way similar to the normal triggering of apoptosis. Finally, IL2-Bax and GnRH-Bak/Bik/Bak represent a novel approach for constructing chimeric proteins by fusing a targeting molecule that binds to a specific cell type to a wide range of apoptosis-inducing proteins. Thus, targeted apoptosis-inducing

chimeric proteins should open up new vistas in the fight against cancer.

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