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Review

Fully Human Targeted Cytotoxic Fusion Proteins: New Anticancer Agents on the Horizon

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Abstract. *Cytotoxic fusion proteins for tumor therapy are composed of an antibody-based targeting moiety and an effector molecule. Effectors may possess enzymatic activity confering cytoxicity after internalization or be an antibody-targeted death-receptor ligand that induces apoptosis after interaction with a death receptor. In this review, we focus on cytotoxic fusion proteins which, in most cases, are composed of fully human targeting and effector moieties. Regarding the first category, as outlined above, we focus on fusion proteins based on ribonucleases, granzyme B, apoptosis-inducing factor and death-associated protein kinases. The second category of fusion proteins makes use of cell-death inducing ligands such as tumor-necrosis factor, tumor necrosis factor α-related, apoptosis-inducing ligand fas ligand and a tumor-targeting antibody moiety. For the latter category, prodrug-related concepts are also covered. The critical issues to be resolved for improved efficacy and safety are discussed.*

Engineered fusion proteins as anticancer agents are clinically evaluated in the context of antibody-directed enzyme prodrug therapy (ADEPT), as ligand traps and as fusion proteins with a cytotoxic protein-based function. For ADEPT and cytotoxic fusion proteins, an enzyme or a death-receptor ligand (DRL) are fused to an antibody-based targeting moiety. An example of a ligand-trap is Aflibercept, a fusion protein consisting of modules derived from vascular endothelial growth factor (VEGFR1) and VEGFR2. Aflibercept binds to several isoforms of VEGF, neuropilin and placental growth factor (PlGF) as an inhibitor of tumor-related angiogenesis (1) and is currently clinically being evaluated for several tumor indications. ADEPT makes use of pre-targeting tumors with antibody-enzyme fusion proteins followed by administration of an inactive prodrug that is converted into its active form by the pre-targeted enzyme (2). Fusion proteins with human or bacterial carboxypeptidases have been assessed in clinical studies for activation of methotrexate or nitrogen mustard at the tumor (2).

Genetically engineered immunotoxins (ITs) are generally made up of an antibody-based targeting module fused to bacterial or plant-derived toxins. As toxin components, Pseudomonas exotoxin (PE), diphtheria toxin (DT) and other ribosome-inactivating proteins such as ricin, saporin and gelonin, have been evaluated in clinical studies (3-5). ITs require internalization mediated by the antibody moiety and endocytosis for efficient release into the cytoplasm by endosome-escape-mediating modules. ITs are able to kill tumor cells efficiently, but combination of target and non-target mediated side-effects and immunogenicity have so far hampered their clinical breakthrough. In particular, a high degree of immunogenicity prevents their long-term application. This makes it necessary to apply rather high doses to achieve efficacy, which in turn leads to undesired toxic side-effects. Therefore, a combination of fully human sequences for the antibody based-targeting module, as well as for the cytotoxic function or the cell death-inducing ligand, might represent an attractive alternative towards application as anticancer agents. Fully human cytotoxic fusion proteins are the focus of this review.

Antibody–enzyme–based Cytotoxic Fusion Proteins – General Issues

Antibody-enzyme fusion proteins must be internalized, must escape from the endosome and eventually become processed for delivery of their cargo into the cytoplasm. Incorporation of cell penetrating peptides (CPPs) into the fusion proteins might enhance internalization, but CPP-mediated membrane transduction might also occur in cells in which the target

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antigen is not expressed. This may cause off-target side-effects. A variety of small peptides such as α- and β-helical peptides, polylysine, polyarginine and peptides enriched in basic amino acids, have been described. The most prominent are TAT (transcriptional activator protein) derived from human immunodeficiency virus (HIV), penetratin, derived from *Drosophila melanogaster* homeotic transcription protein Antennapedia, and herpes simplex structural protein VP2 (6). Internalization of the fusion proteins is mediated by endocytosis (7). Receptor-mediated internalization of ligands such as antibodies and growth factors occurs in most cases by clathrin-mediated endocytosis, starting with clathrin-coated pits and finally as clathrin-coated vesicles with a diameter of 100 nM in the plasma membrane. Glycosylphosphatidylinositol (GPI)-anchored proteins are internalized by a clathrin-independent, cholesterol-dependent endocytic pathway with involvement of caveolin as vesicles with a diameter of 50 nm, enriched in cholesterol and glycolipids (8). Phagocytosis refers to the engulfment of whole particles, whereas pinocytosis makes use of dissolved substrates with no specificity for the substrates being transported (8). A special form of pinocytosis is macropinocytosis, involving the formation of large endocytic vesicles (macropinosomes) generated by actin-driven circular ruffles of the plasma membrane. Macropinocytosis occurs by invagination of the cell membrane forming a pocket which pinches-off into the cell, forming vesicles (0.5-5 μm in diameter) which then fuse with endosomes and lysosomes (8). Dendritic cells which present antigens to T-cells take up antigens by macropinocytosis. Small G proteins of arf, rab and rho families are involved in endocytic processes acting from the cytoplasmic side of vesicles. They promote endocytosis through affecting membrane curvature changes dependent on their intrinsic GDP/GTP loading status (membrane-bound and cytosolic cycles). The endocytic pathway internalizes molecules from the plasma membrane, which can be recycled back to the cell surface or are sorted for degradation. In the early endosomes, the ligands dissociate from their receptors due to the acidic pH in their lumen, and the late endosomes often contain many membrane vesicles; the last compartment of the endocytic pathway are the lysosomes which contain hydrolytic enzymes (9, 10). A bottleneck for the delivery of the enzymatic function of cytotoxic fusion proteins into the cytoplasm is prevention of or escape from degradation in lysosomes and the release from the endosome which often occurs accidentally rather than intentionally. In most cases, pH shifts in endosomal compartments alter conformation and expose processing and routing signals. These processes prevent degradation in lysosomes and direct the toxins to compartments that enable translocation to the cytoplasm. As will be discussed later in this review, routing and processing of cytotoxic antibody-enzyme fusion proteins can also be aided by insertion of sequences designed for routing and processing in the endosome, thus mimicking the processes that have evolved in pathogens.

Immuno-RNAses

An untargeted frog-derived RNAse (Onconase, Ranpirnase) is presently being evaluated as a single agent in a phase III study in patients with unresectable mesothelioma (11, 12). With a few exceptions, such as Onconase, extracellular RNAases are non-toxic. Therefore, RNAses have been fused to targeting moieties to achieve tumor-specific internalization (13-20). Even though immunogenicity seems not to be a major issue for frog RNAse so far, human-derived RNAses would be preferred for therapeutic applications. Among the human RNAses, eosinophil-derived RNAse, eosinophil-derived neurotoxin, angiogenin and human pancreatic RNAse (hpRNAse) were experimentally evaluated in the context of fusion proteins. As tumor-specific targets, clusters of differentiation (CD22, CD30), human epidermal growth factor receptor 2 (HER2), mucine-1 (MUC1), transferrin receptor (TfR) and human placental alkaline phosphatase (PLAP) were chosen most frequently (13-20). Monovalent as well as bivalent antibody fragments were fused to the *N*- or *C*-termini of RNAses. Popular formats are scFv-RNase, RNAse-scFv, dimerized scFv-RNase and scFv-CH^2-CH^3-RNase fusion proteins [(5) and Figure 1A, 1B, 1D and 2B]. Bivalency improves targeting to the tumor site (21), probably due to affinity and valency effects. Comparison of dimeric *versus* monomeric versions of anti-CD22-RNAse fusion proteins showed markedly superior cytotoxicity of the dimeric version towards $CD22^+$ tumor cells (22, 23). Formats including CH^2-CH^3 domains may enhance potency due to activation of complement-dependent cytotoxicity (CDC) (15).

In order to eliminate tumor cells, immuno-RNAses have to be internalized, released from the normal endocytic degradation pathway into the cytosol, evade the cytosolic RNAse inhibitor (RI) and finally degrade RNA, which results in apoptosis. Although the targeted cytotoxicity observed in many examples proves that the RNAses enter the cytoplasm, the exact mechanism by which they escape endosomes is so far unknown. Effective release of the RNAses from the targeting moieties appears to play an important role for endosome escape (see below). Cytotoxicity has been shown to be independent of the p53 status of the tumor cells. Inhibition of catalytic and cytotoxic function by cytosolic RI, a leucine-rich ankyrin-repeat protein that strongly binds to mammalian RNAses in the femtomolar range, and limited release due to endosomal sequestration are critical issues. In addition to degradation of tRNA and rRNA, binding and cleavage of double-stranded (ds) RNA result in altered gene expression, affecting cell viability due to reduced protein synthesis and activation of mitochondrial caspases (24).

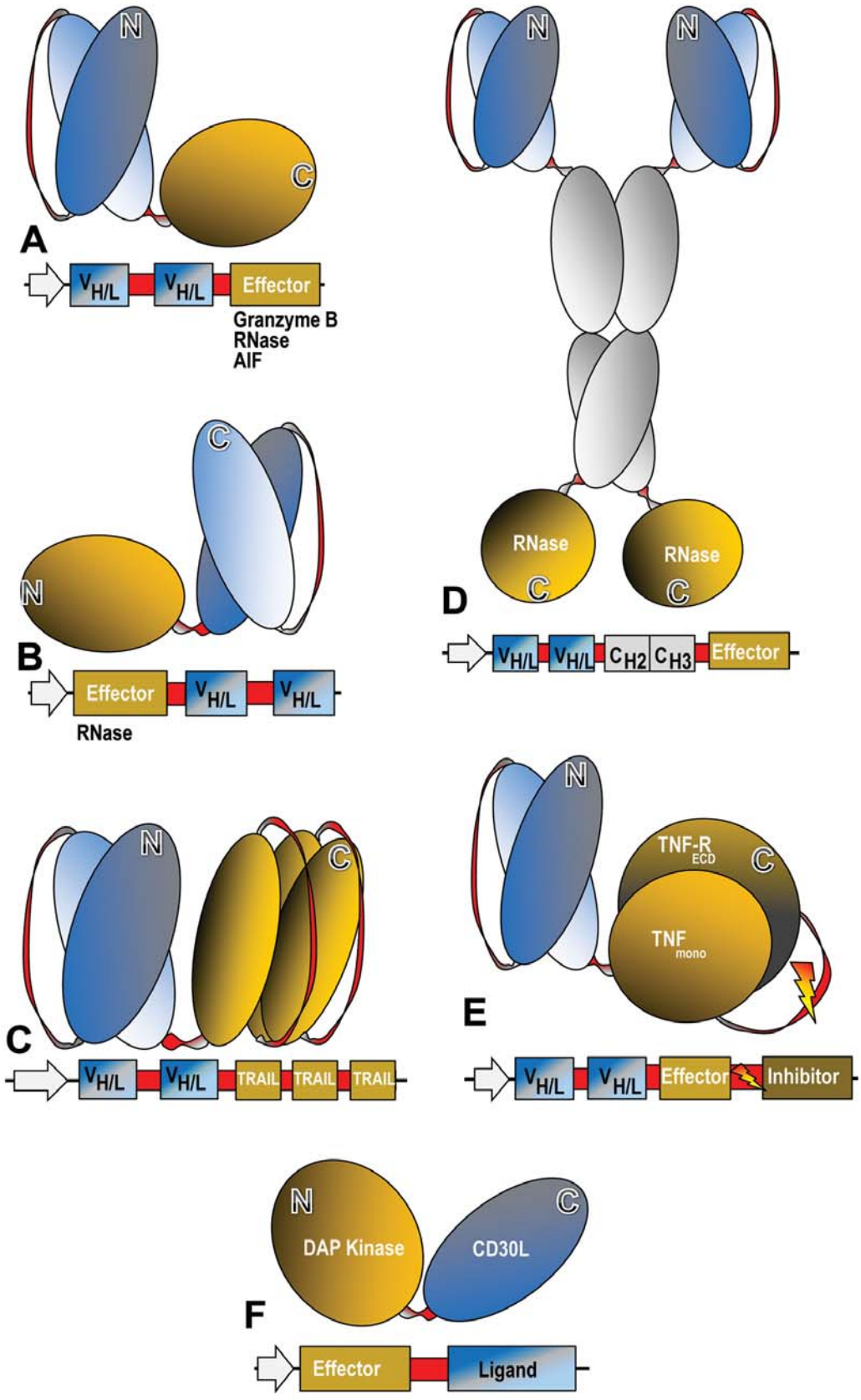
Adding to the complexicity of the mode of action (MOA) of immuno-RNAses it has been shown that catalytically inactive forms of human pancreatic RNAses can induce apoptosis (25). Suppression of protein synthesis is involved in the MOA of immuno-RNAses, but does not seem to be sufficient for cytotoxicity (26). (IC_{50}s) in the range of 0.05 nM and 100 nM have been noted for the most potent immuno-RNAses. The first entirely human fusion protein between anti-HER2 scFv and human pancreatic RNAse (Figure 1B and 2B) mediated *in vitro* cytotoxicity in the range of 12.5 and 60 nM in HER2-positive cell lines and a 86% growth inhibition of syngeneic $HER2^+$ tumors in *HER2/neu* transgenic mice (27). Subsequently, evaluation of several HER2-targeted immuno-RNAses has been described (28, 29). A single-chain fragment of anti-CD64 was fused to angiogenin as a fully human IT and is designed for the treatment of $CD64^+$ malignancies, such as acute myeloid leukemia (AML) (30, 31). An adapter containing a synthetic translocation domain flanked by proteolytically cleavable endosolic and cytosolic consensus sites was incorporated between the antibody moiety and angiogenin (31). The cytosolic consensus site contains caspase-1 and -3 cleavage sites and the endosomal site contains two furin cleavage sites derived from PE and DT. As a membrane translocation motif, a 12 amino acids peptide derived from the Pre S2-domain of hepatitis B virus (HBV) was used. Insertion of the adapter increased the cytotoxicity for U937 cells by up to 20-fold. However, serum stability was markedly reduced. A modified adapter variant lacking the endosomal cleavable peptide showed three-fold higher cytotoxicity compared to the adapter-free IT. Whereas the molecule containing the full-length adapter is nearly completely cleaved in human serum within one hour, the fusion protein with no adapter and that with the truncated adapter remained stable with almost no cleavage after 24 hours. These results underline the usefulness and feasibility of IT improvement by incorporation of optimized cleavable adapters. The issue of immunogenicity of the translocation peptide derived from hepatitis B virus protein has to be addressed. In addition to the antibody-fusion proteins, ligand-RNAse fusion proteins with epidermal growth factor (EGF), interleukin 2 (IL2), fibroblast growth factors (FGFs) and human choriogonadotrophin (hCG) are also under preclinical evaluation (13-16).

Granzyme B-based Fusion Proteins

Granzymes are granule-derived serine proteases which are expressed by natural killer (NK) cells and subpopulations of cytotoxic T-cells (32). Five types of granzymes designated as A, B, H, K, and M, have been described in humans. Granzyme B is unique due to its cleavage after aspartate (33). For activation of the enzyme, the dipeptide Gly-Glu is removed by lysosomal dipeptidyl-peptidase I and the new amino-terminus moves into the interior of the molecule and induces an allosteric change of the activation domain (34, 35).

Two models have been discussed regarding the entrance of Granzyme B into cells to be destroyed by NK and activated T-cells: an endocytosis-dependent mechanism of vesicle disruption, and a direct, pore-mediated delivery of the apoptosis-inducing cargo. In the first model, perforin molecules form circular structures by lateral aggregation, thus generating channels for Granzyme B uptake, Granzyme B then leaking into the cytosol through perforin pores before repair has been completed. In the second model, perforin pores trigger local Ca influx stimulating endocytosis. Granzyme B accumulates in endocytic versicles, which may then rupture and release their luminal content into the cytosol. The cytotoxicity of Granzyme B after internalization is caused by several mechanisms. One is the activation of the caspase cascade *via* procaspase activation of executioner caspases, such as caspase 3. This is followed by caspase 7 activation. Another mechanism is caspase activation *via* cytochrome c and procaspase 9 (34, 36). Release of DNA fragmentation factor 40 (also referred to caspase-activated DNAse, DFF40, CAD) from its endogenous inhibitor DNA fragmentation factor 45 (also designated as inhibitor of caspase-activated DNAse, DFF45, ICAD) is due to its cleavage by caspases. This results in oligomerization of DFF40 and its entry into the nucleus where it fragments DNA. Thus signals leading to apoptosis are amplified (37, 38). In the nucleus, poly(ADP)-ribose polymerase (PARP) and nuclear matrix protein are also degraded by Granzyme B (39). Finally, damage to non-nuclear structures, such as mitochondria, resulting in cell death through caspase-independent mechanisms, has been reported as a consequence of exposure of cells to Granzyme B (40).

Several Granzyme B-related immuno-conjugates (Figure 1A and 2A) were studied with the conclusion that multiple issues have still to be resolved to generate therapeutic entities. Among these, the delivery of the cleaved fusion proteins from the endosomal compartment to the cytosol is critical (32). Specific *in vitro* killing for a Granzyme B-scFv fusion protein targeting LeY was observed (41). The toxin and the targeting components were engineered to contain oppositely charged peptide extensions fused together by electrostatic interactions and a disulfide bond between the cysteine residues of both extensions. Treatment of cells with Granzyme B related fusion proteins with EGFR-ligand transforming growth factor α (TGFα) or scFv-HER2 antibody (Figure 2A) resulted in apoptosis as shown by chromatin condensation, membrane blebbing, formation of apoptotic bodies and activation of endogenous initiator and effector caspases. However, in order to achieve killing in the picomolar to nanomolar range, cells had to be treated with the endosomolytic agent chloroquine (42). This indicates that endosome escape is a bottleneck for potency of Granzyme B fusion proteins. Disruption of normal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VH/L
Effector
Inhibitor
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DAP Kinase
C
CD30L
Effector
Ligand

endosomal functionality by chloroquine is required to enable entrance of fusion proteins into the cytoplasm. A fusion protein between Granzyme B and scFvMEL, which is directed against melanoma-related high molecular weight glycoprotein GP240 was shown to kill A375 melanoma cells at an IC_{50} of 20 nM, and inhibited *in vivo* growth of this cell line. Based on a qd5×5 schedule (37.5 mg/kg), the tumor volume in control mice increased from 50 to 1200 mm^3 *versus* 200 mm^3 in the treatment group (43, 44). A Granzyme B-VEGF121 fusion protein was evaluated in PAE endothelial cells transfected with VEGFR1 or VEGFR2 (45). The fusion protein was internalized in cells expressing VEGFR2, not in cells expressing VEGFR1, with IC_{50}s between 10 and 20 nM for its cytotoxic action. IC_{50}s between 1.7 nM and 17 nM have been observed for a fusion protein between Granzyme B and scFv H22, an antibody fragment directed against CD64 (FcγR1), an antigen expressed in AML (46). Targeting and killing of luteinizing hormone receptor (LHR) positive tumor cells was achieved with a fusion protein in which both chains of its ligand human chorion gonadotrophin (hGH) were yoked together and fused to Granzyme B (47). A fusion protein between scFv-HER2 and Granzyme B secreted after transfection of mammalian cells with an expression plasmid has also been been evaluated (48). It was shown that the fusion protein selectively recognized and destroyed HER2- overexpressing tumor cells, both *in vitro* and in nude mice after intramuscular injection of a corresponding expression plasmid.

Further critical issues regarding the development of Granzyme B-related fusion proteins have emerged. The high isoelectric point of Granzyme B results in strong off-target effects and accumulation of Granzyme B in various body compartments. Six basic amino acids were shown to be involved in these interactions such as binding to heparan sulfate (49). These non-specific interactions can be abolished by mutagenesis of these residues (50). Another critical issue of Granzyme B is its binding to intracellular serpins, especially PI-9, resulting in blocking of their enzymatic activity (51). The ideal Granzyme B-based immunoconjugate would target a receptor with high density and specificity on tumor cells, would be endocytosed, the targeting moiety would be removed by an endogenous protease and the activated Granzyme B translocated into the cytosol with the help of an optimized translocation peptide or domain. Presently, the effective translocation domains are derived from plants, bacteria and viruses, resulting in potential immunogenicity. In addition to mediating little or no immunogenicity, the optimal translocation domain should by active at the lower pH of the endosomal/lysosomal compartment.

Death-associated Protein Kinase (DAPK)-based Fusion Proteins

The DAPK family (ser-thr kinases) consists of DAPK1, DAPK2, DAPK3 (ZIP) and DAPK related protein kinases 1 and 2 (DRAK-1 and DRAK-2) (52, 53, 54). DAPK1 and DAPK2 share a highly conserved *N*-terminal kinase domain and a calcium/calmodulin regulatory domain that controls their catalytic activity by sensing intracellular Ca levels. The unique autoregulatory mechanism of DAPK1 and DAPK2 is based on autophosphorylation, which is relieved when Ca activated calmodulin binds to the calcium/calmodulin domain (55). Therefore, deletion of this region results in mutants such as DAPK2Δ73 with constitutive kinase activity enhancing pro-apoptotic and autophagic signals (56). Ectopic expression of DAPK1 and DAPK2 leads to induction of apoptosis, as shown by membrane blebbing and formation of autophagic vesicles (57). Caspase-dependent and –independent induction of apoptosis mediated by DAPK has been reported (58). Tumor suppressor gene-related properties of DAPK were also unveilled (58). Hypermethylation of the promoter of DAPKs and loss of heterozygosity (LOH) were identified as mechanisms for down-regulation of gene expression (58).

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Figure 1. *Design and composition of cytotoxic fusion proteins. The domain compositions of fusion proteins are shown schematically in the upper panels. Sequences that encode these entities are summarized in the lower panels of each Figure. The general principle for design of cytotoxic fusion proteins includes recombinant fusion of cell surface-targeting modules (blue) to modules that mediate cytotoxicity (brown). Flexible linker or connector sequences are frequently incorporated at the fusion sites of these different entities (red). These sequence-stretches in many cases contain serine and glycine residues to permit flexibility. A: Examples of fusion proteins that have cytotoxic entities fused to the C-termini of a single-chain variable fragment (scFvs) include as payloads granzyme B, RNAse or apoptosis-inducing factor (AIF). B: Some entities, such as RNAses, may also be fused to the N-termini of sc Fvs. C: In cases where multimerization is necessary for cytotoxic activity, effector domains can be connected via flexible linkers. The example shows the composition of a Fv-fusion protein that contains a 'trimeric' tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) module. D: Constant regions of antibodies (grey) can be incorporated into scFv-fusions, e.g. those containing RNAses as payload, to modulate pharmakinetic properties. E: A modification of the scFv-effector format is the addition of releaseable entities that inactivate the toxic payload. In this example, a tumor necrosis factor α (TNFα)-binding domain has been added to the C-terminus of a scFv-TNFa fusion protein. This domain, which neutralizes the rather high nonspecific activity of TNF, is released at tumors by proteases that cleave the linker by which it is attached to the cytotoxic moiety. ECD: Extracellular domain. F: Ligands such as CD30L can be utilized as alternatives to antibody-derived targeting modules. A fusion protein with death-associated protein kinase (DAPK) is shown.*

In the context of antibody DAPK fusion proteins, constitutively active DAPK2 has been explored (59, 60). DAPK2 is expressed in normal tissues such as skeletal muscle, colon, spleen, heart and lung, as well as in leukocytes, but expression in Hodgkin lymphoma cell lines is blocked due to promoter methylation (60, 61). Expression of a fusion protein consisting of CD30 ligand fused with DAPK2Δ73 (Figure 1F, 2C) demonstrated its ability to kill CD30-expressing Hodgkin's lymphoma cell lines with high specificity and IC50s ranging between 7 and 17 nM (9). Impact of the immunokinase fusion protein DAPK2Δ73 CD30L on survival of severe combined immunodeficient (SCID) mice inoculated with L540 Hodgkin's lymphoma cells was demonstrated (60). Mice were treated with the maximum tolerable dose of 70 μg fusion protein per mouse one day after challenge with the tumor cells. The mean medium survival times of mice treated with phosphate-buffered saline (PBS) or non-specific immunokinase controle groups (n=10) were 55 and 60 days, respectively, whereas in the DAPK2Δ73-CD30 L treatment group, 8 out of 10 mice were long-term survivors (>175 days). These experiments indicate that restoration of a tumor-suppressor function with an immunokinase can result in therapeutic benefit in a Hodgkin lymphoma-based *in vivo* model (61). However, the MOA of entry of these fusion proteins into the cell and the cytoplasm remains to be clarified. Whether this concept can be extended to other tumor models remains to be seen.

Apoptosis-inducing Factor (AIF)-based Fusion Proteins

AIF is a mitochondrial flavoprotein with NADH oxido-reductase activity which catalyses electron transfer in the mitochondrial respiratory chain (62). In response to lethal signaling, AIF is cleaved by calpains and/or cathepsins to a 57 kDa protein and is released from the mitochondrial intermembrane space into the cytoplasm from where it translocates into the nucleus and interacts with cyclophilin A to form an active DNAse, whereby the positively charged surface of AIF facilitates binding to DNA in a sequence-independent manner resulting in caspase-independent cell death (63, 64). A fusion protein composed of a signal peptide, a single-chain HER2 antibody, a PE translocation domain and a truncated AIF (AIFΔ100) expressed in mammalian cells selectively recognized HER2-overexpressing cells and was endocytosed [(65) and Figure 1A and 2D]. The fusion protein undergoes furin-mediated cleavage of the PE translocation domain in the endosome and releases a C-terminal peptide containing part of the PE translocation domain and AIFΔ100. Immunofluorescence-based analysis showed nuclear translocation of the AIFΔ100 variant in HER2-overexpressing cells. Intramuscular (*i.m.*) administration of a plasmid expressing the fusion protein resulted in suppressed tumor growth and prolonged survival in the HER2-overexpressing SKBR-3 xenograft model. The fusion protein is not cytotoxic to cells expressing the antigen, probably because the lipid bilayer of the endoplasmic reticulum and the Golgi apparatus does not allow access to the cytosol. Recombinant scFv-HER2-AIFΔ100 and a similar fusion protein containing the PE translocation domain as an endosome escape function, exihibited binding to HER2-overexpressing cells and accumulation in intracellular vesicles (66). In the presence of an endosomolytic agent such as chloroquine, the fusion protein with the translocation domain, but not the one lacking it, displayed cell killing activity strictly dependent on expression of HER2. Cytotoxicity was observed only in the presence of the endosomolytic agent. The translocation domain did not facilitate movement of the active AIF fragment into the cytosol, however in the presence of chloroquine, tumor cells were readily killed. Furin-mediated cleavage of the translocation domain leads to escape of a C-terminal AIF-fragment from the fusion protein by endogenous endosomal furin. Microinjection experiments in VERO cells with untargeted AIFΔ100 and fusion protein containing the PE translocation domain revealed typical apoptotic morphology in cells microinjected with AIFΔ100, but not in cells microinjected with the fusion protein. This would argue for the importance of the translocation domain in liberating active AIF-fragment from the endosome. Both types of fusion proteins were found to bind to DNA *in vitro*. Intracellular delivery of targeted AIF-fusion proteins could potentially bypass and overcome inhibitory mechanisms, preventing caspase activation in tumor cells. Some of the critical issues of this approach are the positive charge of AIF resulting in non-specific binding (64), and the potential immunogenicity of the bacteria-derived translocation domain.

Further Approaches for Cytotoxic Fusion Proteins with Enzymatic Effector Functions

Cytotoxic fusion proteins based on caspase 6 have been explored. Expression of activated caspases in tumor cells would bypass situations in which the caspase activation process is inhibited by anti-apoptotic proteins such as X-linked inhibitor of apoptosis (XIAP), inhibitors of apoptosis (IAPs) and survivin (67). Plasmid-driven expression of fusion proteins consisting of a scFv antibody directed against HER2, a translocation domain derived from PE and truncated active caspase 6 was shown to inhibit the growth of HER2-positive SK-BR-3 human xenografts after *i.m.* injection of liposome engrafted adenoviral expression vectors, by intratumoral (*i.t.*) injection of adenoviral vectors or by *i.v.* infection of peripheral blood mononuclear cells (PBMCs) with retroviral vectors expressing the fusion protein (68).

I.m. injection of liposome-encapsulated vectors for the fusion protein significantly prolonged survival time and also inhibited metastasis of a human osteosarcoma cell line to the lungs (69).

Another strategy is based on anti-TfR-avidin (TfR-Av) fusion proteins. In the context of investigations addressing the question whether anti-rat TfR-IgG-Av could be used to deliver biotinylated molecules into cancer cells, it was discovered that an anti-TfR-Av fusion protein exhibits both strong pro-apoptotic activity and the ability to deliver various molecules into cancer cells (70). The pro-apoptotic effect was shown to require both the specificity of the TfR and the avidin moiety. It was shown that the anti-rat TfR-IgG3-Av exists as a non-covalent dimer. Anti-rat TfR-IgG3 alone did not have any inhibitory effect, however, cross-linking with a secondary antibody resulted in an antiproliferative activity comparable with the anti-rat TfR-IgG3-Av. Divalent anti-TfR-IgG was shown to increase the rate of TfR internalization and degradation and to reduce the growth rate of treated cells, indicating that a partial block of iron uptake and TfR down-regulation may be responsible for the pro-apoptotic function of the anti-TfR-Av fusion protein. Anti-human TfR-IgG-Av also conferred cytotoxicity after treatment of U562 cells. Immunogenicity due to the avidin component may be a problem; however, almost everybody has been exposed to chicken avidin by eating eggs and therefore may be tolerant to this oral antigen.

DRL-based Fusion Proteins

Critical common themes regarding the fusion proteins described in the preceding text are endosome release as a bottleneck; use of chloroquine at high concentration, which is unrealistic in a clinical setting due to toxicity issues; and use of non-human endosome escape modules which might result in immunogenicity. Another strategy is the delivery of cytotoxic payloads from outside of the cell as receptor ligands which do not require endosome release as a bottleneck. DR are members of the TNF-receptor (TNFR) superfamily with functions in regulation of survival and cell death, differentiation and immunity (71-82). A total of 18 ligands and 28 receptors have been identified. Almost all of the receptors and ligands are transmembrane proteins (75). Here we focus on TNFα-TNFR1 and -2, Fas Ligand (FASL) (CD95L, APO1), apoptosis stimulating fragment (FAS) (CD95, APO1) and Apo2 ligand (APO2L) [(TRAIL)-DR4 and -5] as the corresponding ligands and receptors. DRs are expressed on many types of tumors and this finding can be exploited to induce regression of tumors by treatment with their cognate ligands. However, they are also expressed in some types of normal tissues, with the consequence of side effects after treatment (75). After receptor ligand interaction, a death-inducing signaling complex (DISC) is assembled through the adapter protein FAS-associated death domain (FADD), leading to recruitment and activation of caspase 8 and/or caspase 10 (4). In the case of TNFR1, first TNFR-associated death domain (TRADD) is recruited as a platform for the assembly of secondary receptor complexes. DL-DR interaction first induces the cell extrinsic pathway of apoptosis; however, a cross-talk with the mitochondria-mediated intrinsic pathway has been identified. The detailed description of these signaling complexes is not within the scope of this review.

TNFR superfamily ligands share a common structural motif, the TNF homology domain (THD), which binds to cysteine-rich domains (CRD) of the corresponding receptors. CRDs are composed of several modules whose variation in number and type confers unique properties on individual receptors. The THDs adopt a classical jelly roll topology, share a virtually identical tertiary fold and associate to form tertiary receptors (71).

DRs can be engaged with recombinant trimerized ligand (TRAIL, TNF, FASL) or with agonistic antibodies binding to the extracellular domain of the receptors with and without the requirement for cross-linking of the receptors (6). An agonistic antibody might mimic the function of the ligand by stabilizing an active conformation of the receptor and recruit the DISC; a bivalent antibody can also induce the formation of complexes between pre-associated receptors and it has been shown that cross-linking of the Fc portion with secondary antibodies can further augment DISC formation (83). The identification of biomarkers indicative of possible responsivity is a major challenge because induction of apoptosis by the appropriate DL is context-dependent as shown in many experimental systems. Testing a panel of 119 cancer cell lines for TRAIL sensitivity revealed that expression of O-glycosylation enzymes might predict sensitivity through their involvement in receptor modification (84). Currently several pro-apoptotic receptor agonists are in phase I/II clinical studies as monotherapy and in combination with other agents such as rhApo2L/TRAIL which targets DR4 and DR5, Mapatumumab (monoclonal antibody targeting receptor DR4), Lexatumumab, Apomab, AMG 655, LBY-135 and CS-1008 (all monoclonal antibodies targeting DR5) (79).

Due to limitations of therapy with the ligands by themselves, we focus on the improvement of anticancer therapy with genetically engineered fusion proteins.

Low-dose TNF induces angiogenesis, whereas a high dose is cytotoxic to endothelial cells, the resulting damage leading to clot formation and vascular obstruction (85). TNF is in clinical use for the treatment of tissue soft sarcoma as isolated limb perfusion, however, broad application in the clinic is prevented by toxicity related to systemic proinflammatory effects (86, 87). Although stable disease has been reported with TRAIL monotherapy in patients with advanced cancer, monotherapy will probably not result in impressive clinical

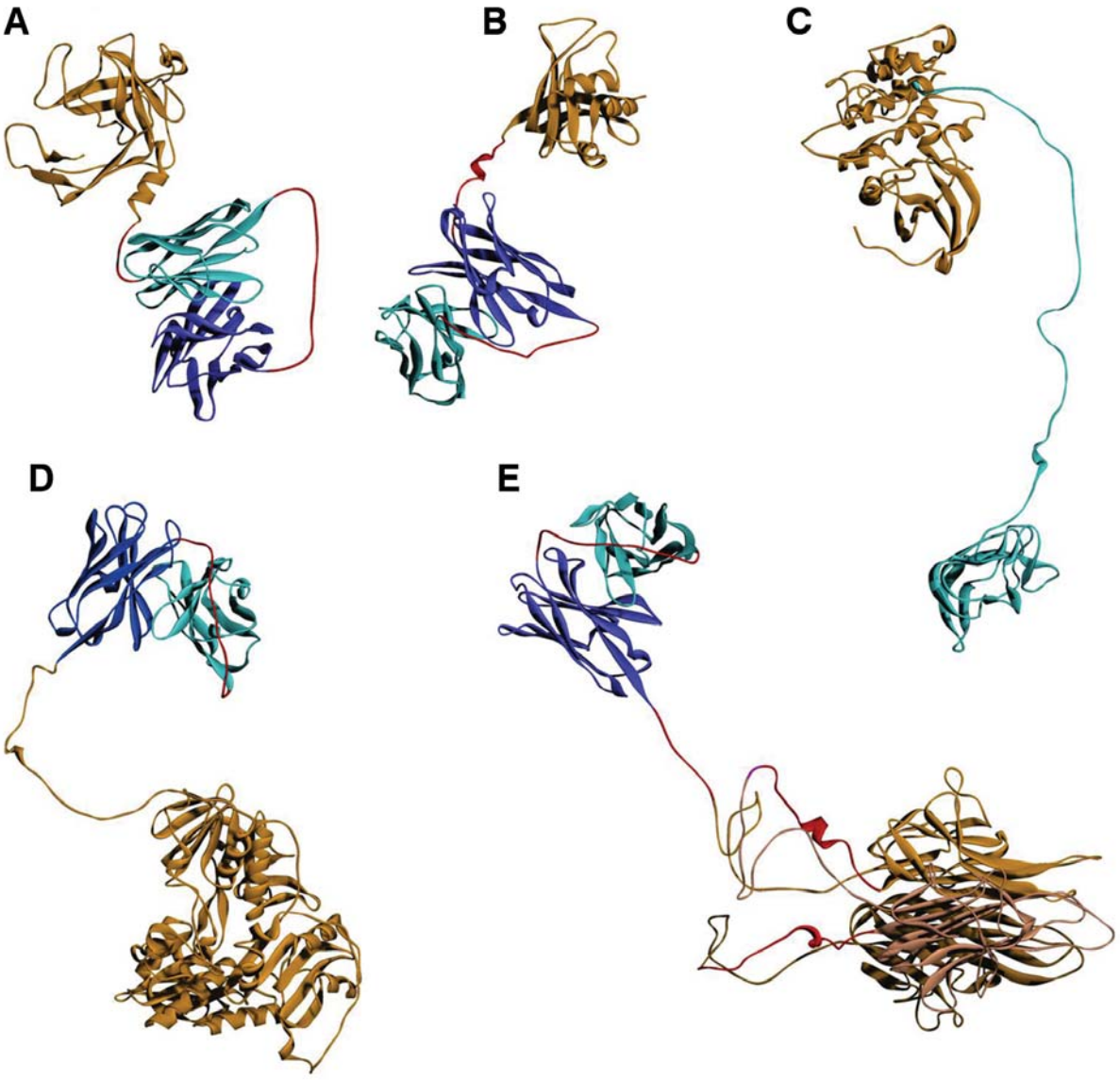

Figure 2. *Structure models of fusion proteins with cytotoxic effectors. The structure models were generated based on available structural data of individual targeting and cytotoxic entities. Structural data from the Protein DataBank (PDB, Nov. 2011) (www.pdb.org) were assembled and minimized using Discovery Studio 31 (Accelrys Software, San Diego). Ribbon representations are shown to facilitate identification of secondary structures where the N-terminal domain is placed on the top and the C terminal domain on the bottom. Linkers that are introduced at the fusion positions of the different entities are highly flexible and hence unstructured. Thus, in contrast to the depicted domain structures and the correctness of the introduced linker lengths, orientation and position of linkers or connections between the domains has been chosen arbitrarily. The color scheme of the models follows that of Figure 1, with targeting modules in blue (dark blue for variable region heavy chain (VH), light blue for variable region light chain (VL)), modules that mediate cytotoxicity in brown, and linker or connector sequences are in red. A: The structure model of human granzyme B (GrB) fused to the HER2 scFv FRP5 is based on the crystal structure of GrBa (pdbcode: 1FQ3) followed by a G4S linker and a homolog Fv structure (pdbcode: 1IAI) to which an 18 amino acid linker (GSTSGSGKPGSGEGSTKG) has been introduced between the C-terminus of VL and the N-terminus of VH. B: Model of the human pancreatic RNAse (hpRNAse) fused to a scFv via a peptide linker originated from Staphylococcus protein A. The hpRNAse (pdb code: 1Z7X) domain is followed by the AKKLNDAQAPKSD linker and a scFv based on the pdb code 1DZB starting with the variable VH linked by a (G4S)3, 15 amino acids and the Vκ. C: Model of the human death-associated protein kinase 2 (DAPK2) fused to the CD30 ligand. The DAPK2 structure (pdbcode: 2YAB) for which the N-terminal 1-283 part is taken (avoiding the calmodulin regulatory region and the 40 amino acid tail) is linked directly to the CD30 ligand; CD30L starts with an 41 amino acid unfolded peptide linker followed by a folded domain built by homology modelling to a TNFα structure (PDBcode: 2ZJC). D: Model of a FRP5 scFv as shown in (A) fused to the human apoptosis-inducing Factor (AIF) for which the first 20 amino acids of the mature AIF form have been built as a flexible linker, followed by the homology model of human AIF based on the murine structure (pdb code: 1GV4). E: Model of HER2 scFv fused to a trimeric tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). A Flag linker, DYKDDDDK, is used to connect the scFv to the first TRAIL unit; the three human TRAIL units (pdb code: 1D4V) each received a 20 amino acid modelled N-terminal linker build from another structure (pdb code: 1P38). Two 20 amino acid (G4S)4 linkers connect the three TRAIL subunits.*

activity. FAS is not or only poorly activated by FAS ligand (FASL), but is activated by membrane FASL (88). Further limitations are the broad expression of DRs on non-transformed cells and the short half-life of the DRL. Therefore repeated injections or continuous infusion is indicated. Despite higher sensitivity of tumor cells to DRL, significant side-effects have been observed. Improvements can be achieved at several levels, such as by targeting of the ligands by fusion with peptides or antibodies, development of prodrugs which are activated at the tumor and improvement of the pharmacokinetic properties.

For the preclinical evaluation of TNF-based fusion proteins it should be kept in mind that human TNF binds to murine TNFR1, but not to murine TNFR2, which seems to be involved in tumor rejection (89, 90). However, only TNFR1 contains a death domain. Fusion of TNF with targeting peptides is one of the approaches for improvement of the therapeutic index. For this purpose NGR-TNFα was prepared by fusing TNFα to the *C*-terminus of the peptide asparagine-glycine-arginine (NGR), a selective ligand of aminopeptidase N (APN, CD13) which is overexpressed in endothelial cells of tumors. NGR-murine TNFα inhibited tumor growth by more than 10-fold better than muTNFα and strongly enhanced the efficacy of several cytotoxics (91, 92). NGR-TNFα is currently being evaluated in several clinical phase I/II studies as monotherapy and in combination with several chemotherapeutic agents.

Another approach for improving the therapeutic index of TNFα is its combination with an antibody-based tumor-targeting module as a fusion protein. As a rule, antibody modules in the single-chain variable fragment (scFv) format are used. In one of the earliest approaches, TNFα was fused at its C-terminus with scFv recognizing the LeY antigen (93). The rationale for fusing scFv to the C-terminus of TNF is its involvement in receptor binding and therefore to attenuate the toxicity of TNF by reducing binding to its receptors; the targeting of the fusion protein to the tumor cells should compensate for the reduced bioactivity of TNFα. It was shown that the fusion protein binds to LeY-expressing cancer cells, but with significantly lower affinity for TNFR1 compared to the TNF trimer. Regression of MCF-7 xenografts in mice at doses which are not toxic was observed. The cytotoxicity is based on induction of apoptosis in sensitive tumor cells by interaction of the fusion protein with its receptor without the requirement for any additional cytotoxicity on cells *in vitro*. Stimulation of lymphocytes by TNFα might also play a role *in vivo* (94-96). Comparison with a LeY-targeted PE toxin-based IT with the same antibody TNFα fusion protein revealed a 10-fold decrease in potency. A scFv directed against the melanoma antigen GP240 was fused to TNF (scFcMEL-TNF). It displayed a 250-fold higher apoptotic activity than human TNFα on GP240-expressing cells. Repeated administration mediated regression of A375 melanoma xenografts *in vivo* (97, 98). Fibronectin B is a fn splice variant exclusively expressed in the extracellular matrix of tumor cells and is therefore an excellent target for recruitment of fusion proteins to tumors, as shown in several preclinical *in vitro* and *in vivo* experiments between fn B specific antibody L19 and TNFα (99). This fusion protein is currently being evaluated in phase I/II studies in patients with sarcoma and colorectal cancer.

Due to its systemic toxicity, administration of TNFα as a prodrug which is specifically activated after reaching the tumor has been investigated (85). The underlying principle is the activation of TNFα by proteases which are overexpressed by tumor cells and/or the microenvironment of the tumor such as urokinase plasminogen activator (uPA), tissue-type plasminogen activator, factor VIIa, thrombin and metalloproteinases. As a target, fibroblast activation protein (FAP), a transmembrane receptor expressed by tumor cells and fibroblasts (100), was chosen and scFv36 was directed against FAP as a targeting module. Furthermore, the observation that the extracellular domain of TNFR1 can function as a TNF inhibitor was exploited. A fusion protein consisting of scFv36, a peptide linker, TNFα, a protease-sensitive linker and a TNFR1 fragment containing a C-terminal myc-His-tag for detection and purification was evaluated (Figure 1E). Subsequently huTNF was replaced by muTNFα for evaluation *in vivo* (101-103). The prodrug was shown to bind to FAP-positive tumor cells and was processed by proteases into TNF variants with membrane-TNF-like activity. A bystander effect affecting FAP-negative tumor cells was also observed.

Immunogenicity of the fusion protein cannot be excluded, keeping in mind that even fully human antibodies can elicit an immune response [human-anti-humanized antibodies (HAHA)] (104). The same principle has been applied to the design of FASL-based prodrugs (105). FAS positive, FAP-transfected HT1080 human fibrosarcoma cells were used as the experimental system for *in vitro* and *in vivo* experiments. The prodrug consists of an *N*-terminal scFv directed against FAP, followed by the extracellular domain of FASL, a protease-sensitive linker which can be cleaved by (MMP2) and related proteases and a carboxy-terminal domain corresponding to the extracellular domain of FAS. In order to achieve tumor-associated activation of FAS, FASL is only unmasked after binding of the fusion protein to FAP-positive tumor cells and subsequent cleavage by tumor-associated proteases such as MMPs and uPA; the released scFv-FASL stays membrane-bound thus mimicking membrane FASL. It was shown that activation of FASL *in vitro* is dependent on FAP binding and MMP-mediated processing. Local injection of FAP-transfected HT1080 cells with the prodrug one day after injection of 1.5×10^6 cells resulted in 80% tumor growth inhibition and not in FAP-negative HT1080 cells. It would be of interest to evaluate whether efficacy can be

demonstrated after *i.v.* administration of the prodrug and to evaluate dose-dependent *in vivo* efficacy. *In vivo* activity was blocked with inhibitors of MMPs or uPA and with antibodies directed against FAP.

Another approach for improvement of DRL-based anticancer treatment is the combination of an antibody-based targeting module with single-chain variants of the DRL (106). An scTNF composed of three TNF molecules connected by short peptide linkers displayed enhanced stability and antitumoral activity (107). A new format consisting of a scFv directed against HER2 fused to a scFv version of TRAIL was evaluated. The three TRAIL monomers were connected by two peptide linkers containing four repeats of the sequence GGGS [(106) and Figure 1C and 2E]. Expression as a monomer corresponding to trimeric TRAIL was confirmed. *In vitro*, the targeted scFv-scTRAIL fusion protein displayed higher apoptotic activity in comparison to scTRAIL (3- to 5-fold improvement). *In vivo*, the fusion protein was evaluated in HER2-positive Colo205 xenografts. Local and systemic administration demonstrated the superiority of scFv-scTRAIL *versus* scTRAIL with regard to inhibition of tumor growth. However, the tumor volume (25 mm^3) was small at the beginning of treatment and it would be of interest to extend these studies to established xenografts with a volume of ≥100 mm^3 at the start of treatment. The formation of higher order complexes which are toxic to non-transformed cells could be prevented by making use of this format (108). In general, it would be of interest to compare the genetically engineered TRAIL versions with appropriate agonistic antibodies in clinical trials and with corresponding bispecific antibodies regarding their *in vitro* and *in vivo* properties. The half-life of DRL and also those of its genetically modified versions are short, ranging from 6 to 30 min in mice and monkeys (109, 110). The standard method for improving the pharmacokinetic properties, namely modification with polyethylene glycol (PEG) resulted in loss of bioactivity; however, PEGylation of a lysine-deficient TNFα resulted in the maintainance of full bioactivity (111). Human serum albumin (HSA) fused to the N-terminus of DRL apparently does not significantly alter bioactivity and improves retention in the plasma (112).

Trimerization of DRL is mediated by hydrophobic interactions, therefore subunit dissociation and denaturation has been observed. Stabilization was observed on introduction of a second scaffold trimerisation domain derived from tenascin C or an altered leucine zipper (113-115).

Deimmunization

Although the fusion proteins discussed in this review are composed of fully human components, the constituents are connected by linker and adaptor sequences and new junctions between the components are created. Therefore, immunogenicity in humans cannot be excluded. Coupling with high molecular weight PEG is one of the options, however, the success is case-to-case dependent (116, 117). Another approach is to treat patients with immuno-suppressive agents such as cyclophosphamide and fludarabine (118, 119). Alternative approaches are the identification and removal of B- and T-cell epitopes (120-122). Removal of B-cell epitopes has been evaluated for de-immunization of PE and significant reduction of immunogenicity has been observed in preclinical models and in clinical studies (123-126).

Conclusion

For both approaches, targeting tumor cells with cell death-inducing enzymes after internalization and induction of apoptosis by activating cell DRs with their respective targeted ligands, the tumor specificity of the antigen under consideration is an important issue regarding efficacy and side effect profile. For both categories, the antigen density comes into play. For the first category, efficacy of internalization of antigen-fusion protein complexes and recycling of the antigen are important parameters. For enzyme-equipped fusion proteins, efficient endosomal escape is a limiting factor. Translocation sequences derived from bacterial toxins efficiently promoting this process, however, may be immunogenic. Therefore, identification of human translocation domains is a high priority issue in this context. The concept of creating modular adapters consisting of a membrane transfer (translocation) peptide flanked by endosomally and cytosolically cleavable peptides is a topic for further investigation and optimization with respect to the membrane transfer (translocation) peptide and the choice of the specificity of the endosomal and cytoplasmic cleavage sites. Initial experiments have shown that the insertion of an adapter, as described, leads to 10-fold increase in cytotoxicity (31), but has a negative impact on serum stability. Moreover after deletion of the endosomal cleavage site, the fusion protein still mediates a threefold increase in cytotoxicity with maintainance of the serum stability of the adapter-less fusion protein scFv-CD64-angiogenin. Whether case-to-case optimization is necessary, or a generally optimized modular adapter can be designed is an unresolved question. The presence or absence of intracellular inhibitors of the death-inducing enzymatic functions are important modulators of efficacy. For death-inducing fusion protein-based ligands engaging DRs, biomarkers for identification of death-responsive tumors is a high-priority issue. Comparative studies with agonistic antibodies targeting the corresponding DRs and corresponding bispecific antibodies are also mandatory in order to estimate the level of improvement achieved. In most cases, scFv antibody modules which do not confer the pharmacodynamic properties of the fusion

proteins have been used, calling for improvement of the pharmacodynamic properties. For prodrugs of DRL-based fusion proteins, efficacy after *i.v.* administration and treatment of established (≥100 mm^3 xenografts) remains to be demonstrated. The choice of proteases and their corresponding cleavage sequences incorporated into the fusion proteins mediating tumor-specific and not systemic activation in serum is a critical issue. Potential immunogenicity due to newly created junctions and linkers is a theoretical concern which can only be answered case by case after evaluation in patients, however, de-immunization strategies are gaining increasing attention. Further critical issues might be the accumulation of the fusion proteins in the kidney or non-specific membrane interactions based on the translocation domains resulting in kidney, liver or other organ-related toxicities.

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