Journal of Genetic Syndromes & Gene Therapy
ISSN: 2157-7412

Vago, J Genet Syndr Gene Ther 2015, 6:3
DOI: 10.4172/2157-7412.1000272

Review Article Open Access

Ribosome Inactivating Proteins: Exploiting Plant Weapons to Fight Human Cancer

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Abstract

Ribosome inactivating proteins (RIPs) are potent toxins from plant or bacterial origin that work by irreversibly block ribosomes of target cells, causing apoptotic cell death. Their noteworthy activity has been exploited to therapeutic purpose in the treatment of cancer. The specificity for malignant cells has been addressed by using targeting domains such as antibodies to form immunotoxins or ligands to get chimeric fusions. These therapeutics have shown encouraging preliminary results in hematological tumors as well as in solid tumors with the most effective ones currently undergoing clinical trials. Denileukin diftitox, a fusion between the catalytic domain of the diphtheria toxin and human cytokine interleukin-2, was the first toxin-derived drug approved by the FDA. Another, more recent way to take advantage of RIPs is to utilize their DNA in the suicide gene therapy. Toxin DNA can be delivered trough a vector to or complexed and directly injected in the tumor. In the future RIPs are expected in to give a significant contribution in the treatment of the cancer, also in combination with traditional therapies. The scope of this review is to highlight advantages and drawbacks of RIP based biomedical applications, by evaluating their employment as either targeted proteins or genes as effectors.

Over the past decades, toxins have catalyzed great attention and interest in the scientific community for their therapeutic potential and have been extensively investigated in order to be exploited as potent and versatile weapons against cancer. Immunotoxins (ITs) or toxin-based chimeric fusions represent a well-studied way to exploit the activity of a protein toxin specifically delivered to antigens over-expressed on the surface of malignant cells. Among toxins, ribosome-inactivating proteins (RIPs) are a group of potent inhibitors of protein synthesis, produced by different plant species, but additionally found in a number of bacteria, fungi and algae, whose activity has been increased by natural selection over millions of years. RIPs exert their toxic effects through binding to the large 60S ribosomal subunit on which they act as an N-glycosidase by specifically cleaving an adenine residue exposed in the universally conserved tetraloop GAGA of 28S ribosomal RNA. This results in the inability of the ribosome to bind elongation factor 2 (EF-2), thus blocking protein translation and leading to a prompt apoptotic cell death of target cells. The catalytic site is formed by several key amino acid residues highly conserved among RIPs, belonging to the "N-glycosidase signature", that are folded to define a cleft where the target adenine in the substrate is inserted and processed. Besides RIPs, other well-known toxins inhibit protein synthesis at translation level, though with a different mechanism: diphtheria toxin (DT) and Pseudomonas exotoxin A (PEA) directly inactivate EF-2 by ADP ribosylation, thereby inhibiting amino acid chain elongation and causing cell death [1]. A relevant number of formulations derived from toxins in general and RIPs in particular have been demonstrated to be effective in animal models and have been employed in clinical trials with encouraging results [2]. Denileukin diftitox was the first recombinant fusion formed by a truncated form of DT and human cytokine interleukin-2 which the US Federal Drug Administration (FDA) approved for the treatment of cutaneous T-cell lymphomas [3]. This class of molecules has revealed a great potential in the oncological field and many efforts are currently employed in the development of drugable objects. By acting in a cell cycle independent manner, RIPs can target both quiescent and rapidly dividing tumor cells. This feature makes them suitable to contrast both aggressively growing cancers (e.g., melanoma) and tumors with slower progression (e.g., prostate tumors).

Toxins-based recombinant therapeutics for the treatment of cancer

At the moment, toxin-based formulations have been mainly developed against lymphomas and leukemia, since they could be injected into the blood stream and would more easily reach target tumor cells. In addition to the above mentioned Denileukin diftitox, other ITs formed by an antibody to the CD25 subunit of the IL-2 receptor and a truncated form of the PEA or ricin A chain (RTA) have shown encouraging results (10-40% positive response rate) in phase I/II trial for treatment of chemotherapy-resistant leukemia [4,5]. Anti-CD19, -CD22, -CD30 and -CD38 antibodies have been proven to efficiently and specifically deliver plant RIPs (saporin, PAP and momordin) to lymphoma cells in animal models and combination of 3 of them cured severe combined immunodeficient mice injected with the human B-cell lymphoma cell line Ramos, resulting in 100% disease-free survivors at 10 months [6-8]. Recently, leukemia stem cells have been recognized as a suitable target and anti-CD123-PE IT was proven to be cytotoxic in leukemic cell lines [9]. To date, clinical evaluation of the most promising ITs is underway.

Chimeric fusions between RIPs and growth factors whose receptors are over-expressed on the cell surface of cancer cells have been developed and used in the treatment of certain solid tumors as well. Since different tumors share the same targets, toxin-derived drugs have been assayed on more models. This is the case of a fusion between SAP and the

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Received September 12, 2015; **Accepted** October 13, 2015; **Published** October 20, 2015

Citation: Vago R (2015) Ribosome Inactivating Proteins: Exploiting Plant Weapons to Fight Human Cancer. J Genet Syndr Gene Ther 6: 272. doi:10.4172/2157-7412.1000272

epidermal growth factor tested on breast carcinoma, adenocarcinoma and cervical cancers, exhibiting a remarkable reduction of tumor mass in murine models of the disease [10-12]. SAP has been also fused to the basic fibroblast growth factor and showed a significant tumor growth and metastatization inhibition and increased survival time in neuroblastoma, melanoma and prostatic carcinoma xenografts [13-15]. Not only fusion, but also ITs have been utilized in the treatment of solid tumor, even if they did not shown the same successful results obtained with the hematological malignancies, due to scarce accessibility of the tumoral mass and triggering of immune response.

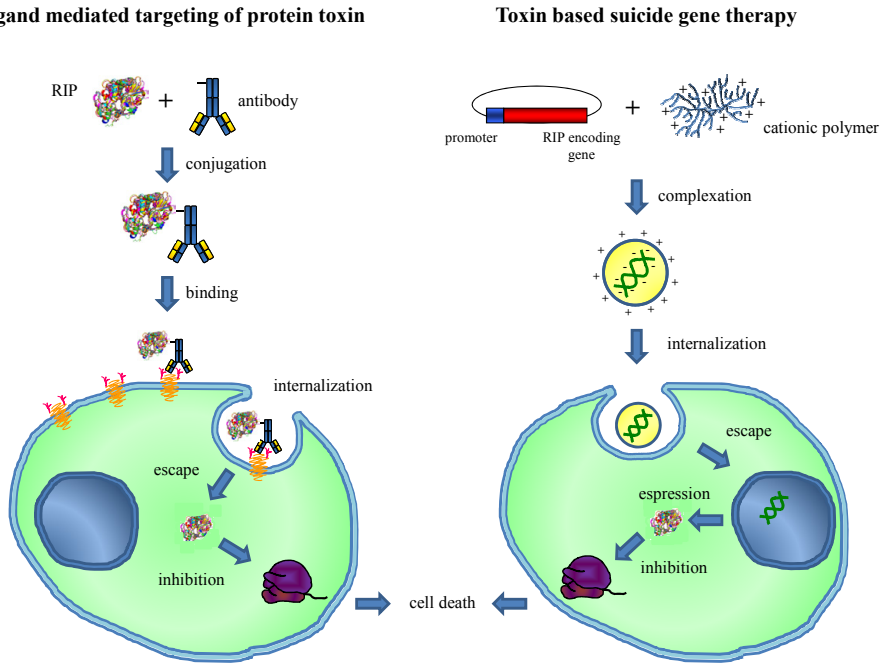
Another promising strategy to kill the tumor is to target the actively growing neoformed vessels associated to the tumoral mass. Several angiogenic factors such as MHC class II, CD44, NCAM and VEGF have been proposed as therapeutic targets as their receptors are over-expressed on the endothelium of tumor vasculature while are almost undetectable in the adjacent normal tissues. A chimeric fusion between the RIP gelonin and the N-terminal fragment of the VEGF demonstrated a reduction in tumor volume in human melanoma or human prostate xenografts, by causing thrombotic damage [16] .

A major drawback when using murine antibodies as targeting domains, as well as plant or bacterial toxins as therapeutic agents, is represented by their potential immunogenicity after repeated administrations. The development of ITs containing fully humanized antibodies or antibody fragments lacking Fc portions in part avoided immunogenicity problems. However, bacterial toxins such as DT or PEA elicit anti-toxin responses upon multiple treatments, also due to worldwide vaccination programs, more prominent with respect to RIPs from plant origin (ricin, saporin, gelonin, etc.) and require to be deimmunized through the substitution of critical amonoacidic residues. Up to now, a great effort of deimmunization has been performed for PEA with convincing results [17]. Another dangerous side-effect observed during IT clinical administration regards the unspecific binding of the toxin domains to the vascular endothelial cells, causing the so-called "vascular leak syndrome". The exact molecular mechanism is not completely understood, but it appears that a consensus sequence present in several RIPs is responsible for binding and vascular damage of endothelial cells. Replacement of critical amino acids was also aimed at abolishing this side effect, while preserving the catalytic activity [18].

The production process of a toxin-based drug requires plenty steps, first of which is the choice of the most appropriate expression system. Since a universal host is still not available, bacteria and yeast are currently the most utilized organisms. One common problem in the production of recombinant RIPs resides in their intrinsic toxicity toward the host ribosomes. To overcome the problem, in bacteria, the toxin expression can be tightly regulated by employing specific *E.coli* strains to partially limit the self-intoxication while getting a satisfactory yield. However, in some cases the protein may accumulate inside the cell as an insoluble fraction from which the full RIP activity is not easily recovered. Several targeting domains belonging to chimeric fusions require complex post-traslational modifications to reach their native conformation and thus they have to be produced in eukaryotic expression systems, like the methylotrophic yeast *Pichia pastoris* that also allows safe, efficient secretion of the heterologous protein in the culture medium followed by an easier purification [19].

Toxin-based suicide gene therapy

The cytotoxicity of all these compounds can depend on multiple factors, like the tumor accessibility and penetration and the efficient internalization, intracellular sorting and release of catalytically active



Dual strategy in RIP-based therapy.
To confer target selectivity, RIPs can be directly conjugated to antibodies or part of them (left) or, alternatively, expressed as chimeric fusion with specific ligands. The antibody recognizes a protein over-expressed on the malignant cells and the immunotoxin is internalized. The toxin is able to escape directly from the endosomal compartment or follow the retrograde transport along the secretory route. Once in the cytoplasm, it blocks the ribosome activity, causing cell death. Alternatively, RIP encoding DNA can be employed in the suicide gene therapy (right). Toxin gene can be complexed by cationic polymers or delivered by vectors. Complexes are internalized and DNA reaches the nucleus where it is transcribed. The protein toxin is produced directly in the cytosol, where it exerts its lethal function.

toxin domains into the cytoplasm, where it exerts its inhibitory activity. In some cases, as for ricin or PEA, the process includes the import into the endosomal vesicles, retrograde transport along the secretory route and retrotraslocation into the cytosol. Other toxins, such as DT or SAP are likely invading the cytosol directly by the endosomal compartment. Another important aspect to be considered, resides in the resistance acquired by cancer cells caused by either loss or down-regulation of specific receptors by autocrine ligands which, thus, reduce binding and internalization of the recombinant toxin. This explains why several promising toxin-derived drugs did not demonstrated great efficacy in clinical trials.

Short while ago, to surmount some of these limitations of recombinant protein mediated approach, DNA-based suicide therapies have been developed. As an effector, the plasmid DNA holds many positive aspects compared to proteins. Indeed, the DNA production and purification is less costly and time-consuming even at large scale; plasmid DNA does not trigger immune system response or vascular leak syndrome, even after repeated administrations; low doses are active since very few molecules of the toxin generated in the tumor cells are sufficient to kill them; tumor cells do not develop resistance to the drug.

As for every drug, the specificity is a key element in the development of plasmid DNA as therapeutic agent. Different safety levels can be introduced and, in particular, working at DNA level allows the direct screening of a wide variety of constructs, employing inducible or tumor specific promoters and transcription regulatory elements, to prevent detrimental expression of toxin in inappropriate tissues. Two strategies are employed to deliver the suicide gene carrying plasmid: recombinant disarmed viruses or non-viral vectors. Both approaches boast undoubted advantages, but also are subjected to constraints; as long as an ideal system for all-purposes has not been identified, the selection of the delivery vector should be established according to the requirements of the specific disease to be treated.

So far, restricted panels of toxins have been used in cancer suicide gene therapy. DT is one of the best studied therapeutic agents and it is a landmark for the toxin gene therapy, since it has been delivered through viral and non-viral vectors. DT has been demonstrated to be effective in many cellular and animal models and is presently under investigation in clinical trials [20]. Recently, two phase I/II clinical trials are performed involving H19 tumor promoter driven DT complexed with the synthetic polycation polyethylenimine. The complex has been administrated intratumorally in patients with unresectable, locally advanced pancreatic cancer or with superficial bladder cancer, who have failed previous therapy with the Bacille Calmette-Guérin [21]. Direct intratumoral injection of saporin encoding plasmid DNA complexed with the cationic lipid DOTAP has been proven to attenuated tumor growth in a mouse model of melanoma, with an effect that was improved upon repeated administrations [22]. Retroviral vectors bearing RTA or PEA genes were able to eradicate experimentally induced brain tumor in Wistar rats [23].

The therapeutical use of cationic polymers was until now mainly confined to intratumoral administration, due to the considerable reduction in their delivery activity by serum components and to some toxicity following systemic administration. To tackle those issues, hydrophilic masking agents like polyethylene glycol have been inserted, increasing the circulation time in blood stream and reducing the liposome toxicity. In addition, the biological activity and specificity to target cells of plasmid DNA have been enhanced by the association of proteins, antibodies, peptides or other agents. As an example, basic FGF was shown to efficiently deliver DNA complex encoding saporin to target cells, leading them to death [24].

In the last years, nanoparticle-based delivery systems have also emerged as potential gene carriers due to unique properties of a nano-scale matter, the diversity of available materials and infinite design schemes. They have been shown to reduce the systemic side effects due to their low toxicity; to increase the therapeutic effectiveness of drugs, by sustained drug release and to accommodate large DNA plasmids or RNA. Nanoparticles spontaneously accumulate in solid tumors after intravenous administration, owing to an increased permeability of tumor blood vessels and a decreased rate of clearance within the tumor (the so-called Enhanced Permeability and Retention effect). In addition, they can be easily functionalized, acquiring selective targeting toward molecules on cancer cells.

In conclusion, targeted delivered RIPs have shown a huge potential in cancer treatment, making them suitable and eligible in the next future for further clinical applications in patients, also in combination with surgery, chemo or radiotherapy. The incessant assessment and identification of novel, early tumor markers will be of great importance to increase the specificity and efficacy of RIP-based drugs. The development of methods as the photochemical internalization or the use of saponins as adjuvant to enhance the cytotoxicity, by specifically acting on their internalization and delivery routes and favoring cytosolic escape, will allow the broadening of the therapeutic windows together with a simultaneous dose lowering.

Acknowledgements

This work was supported by the Italian Ministry of Health (grant GR-2011-02351220)

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