

Review

Gene therapy for carcinoma of the breast Genetic toxins

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Received: 3 November 1999
Revisions requested: 23 November 1999
Revisions received: 25 November 1999
Accepted: 26 November 1999
Published: 17 December 1999

Breast Cancer Res 2000, **2**:22–27

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Abstract

Gene therapy was initially envisaged as a potential treatment for genetically inherited, monogenic disorders. The applications of gene therapy have now become wider, however, and include cardiovascular diseases, vaccination and cancers in which conventional therapies have failed. With regard to oncology, various gene therapy approaches have been developed. Among them, the use of genetic toxins to kill cancer cells selectively is emerging. Two different types of genetic toxins have been developed so far: the metabolic toxins and the dominant-negative class of toxins. This review describes these two different approaches, and discusses their potential applications in cancer gene therapy.

Keywords: cancer, gene therapy, suicide gene

Introduction

The first demonstration of the use of controlled expression of an exogenous gene encoding a toxin as a mean of killing cancer cells was described in 1986 [1*]. In those experiments, a direct suicide gene (the diphtheria toxin A chain) was used. Extrapolated to a clinical scenario, the use of such a potent genetic toxin (a single diphtheria toxin A chain molecule is capable of killing a cell) would require efficient and very reliable selective targeting of cancer cells. This targeting can be achieved by targeted delivery [2] or by transcriptional targeting [3]. For breast cancer gene therapy, a few promoters have already been used to drive the expression of genetic toxins (Table 1). With current tools, however, accurate targeting cannot be achieved. To accommodate these safety issues, a whole branch of cancer gene therapy has been dedicated to the design of more controllable and specific genetic toxins. These new 'suicide' genes can be classified into two groups: the metabolic suicide genes and

the dominant-negative class of suicide genes. This review describes the rationale of these suicide systems and presents their potential applications in breast cancer therapies.

Metabolic suicide gene systems

This strategy relies upon intracellular conversion of a relatively nontoxic prodrug into a toxic drug by an enzyme of xenobiotic origin, and has been referred to as genetic prodrug activation therapy (GPAT). Plant, fungi, bacteria and viruses often utilize unique metabolic pathways that are adapted to their life cycles and environments. These metabolic routes are not used by mammalian cells. In the case of pathogen infections, the distinctive enzymes responsible for these functions have been the target of prodrugs that are developed to be selectively metabolized in infected cells, leading to their destruction. This process is selective, because the prodrug is not toxic to healthy, uninfected mammalian cells.

Table 1

Promoter constructs that have been exploited for breast carcinoma gene therapy

Promoter construct	Reference
DF3/MUC1 promoter	[37]
<i>ErbB-2</i> promoter	[3,27*,38]
<i>ErbB-2</i> promoter/MUC1 enhancer	[39]

The transfer of the genes encoding these enzymes to mammalian cells is sufficient to confer sensitivity to particular prodrugs that are metabolized by the individual enzymes. In terms of cancer gene therapy, the process would involve targeted delivery of these genes to the cancer cells, followed by the administration of the prodrug. Compared with a more direct approach, using a suicide gene such as the diphtheria toxin A chain, this prodrug-enzyme system offers extra levels of control, in terms of variation of prodrug concentration as well as prodrug bioavailability and tissue distribution.

Examples of enzyme-prodrug systems

The most suitable enzymes are monomeric proteins, without any requirement for glycosylation. More complex enzymes may not be correctly folded in an ectopic environment and, as a result, may be less efficient at converting the prodrug. This conversion should be rapid [high coefficient of catalysis (K_{cat})] and should require low concentrations of prodrug [low Michaelis-Menten constant (K_m)]. In addition, the prodrug should be at least two orders of magnitude less toxic than the active anabolite.

Several enzyme-prodrug systems have been reported (Table 2), and some of these are described below.

Herpes simplex virus thymidine kinase

Ganciclovir and acyclovir are guanosine analogues that are poorly metabolized by mammalian cellular thymidine kinases. By contrast, herpes simplex virus thymidine kinase (HSV-TK) metabolizes these prodrugs very efficiently to their monophosphate forms (the rate-limiting step). In turn, the monophosphate form is metabolized to ganciclovir diphosphate and triphosphate by cellular enzymes. The triphosphate form of the prodrug inhibits α -DNA polymerase [4] and is incorporated into DNA, resulting in chain termination during replication [5]. This molecular mechanism of action implies that this enzyme-prodrug system will only be effective for actively dividing cells and should not affect quiescent cells within a tumour. This view has been challenged by the observation that this system induces significant cell death in tissues with low mitotic indices, however [6]. In these cases, the mechanism of action is unclear and seems to involve a p53-independent apoptosis [6]. Very recently, a library of mutants of HSV-TK was created, from which a more effective form of the enzyme (mutant 30) was described [7*]. This type of approach could be applied to improve the characteristics of other enzymes.

Cytosine deaminase

Cytosine deaminase (CD) is an enzyme of bacterial or fungal origin that is activated in response to nutritional stress, deaminating cytosine to uracil. This enzyme became a target for therapy and the prodrug 5-fluorocytosine was selected. 5-Fluorocytosine is metabolized to

Table 2

Enzyme-prodrug systems under investigation for genetic prodrug activation therapy applications

Enzyme	Prodrug	Cytotoxic product
Herpes simplex virus thymidine kinase	Ganciclovir	Ganciclovir triphosphate
Cytosine deaminase	5-Fluorocytosine	5-Fluorouracil
Varicella zoster virus thymidine kinase	6-Methoxypurine arabinose	Adenine arabinonucleoside triphosphate
Nitroreductase	CB1954	5-Aziridinyl-4-hydroxylamino-2-nitrobenzamide
Cytochrome p450	Cyclophosphamide	Acrolein and phosphoramidate mustard
Thymidine phosphorylase	5'-Deoxy-5-fluorouridine	5-Fluorouracil
Purine nucleoside phosphorylase	6-Methylpurine-deoxyriboside	6-Methylpurine
Alkaline phosphatase	Etoposide phosphate	Etoposide
Carboxypeptidase A	Methotrexate-alanine	Methotrexate
Carboxypeptidase G2	Benzoic acid mustard-glucuronide	Benzoic acid mustard
Linamarase	Linamarin	Cyanide
Xanthine oxidase	Xanthine	Oxygen radicals
β -Lactamase	Cephalosporin-mustard-carbamate	Nitrogen mustard

5-fluorouracil by CD. Further metabolism of 5-fluorouracil to 5-fluorouridine-5'-triphosphate and 5-fluoro-2'-deoxyuridine-5'-monophosphate results in cell death by affecting RNA and DNA synthesis. The cytotoxic action of CD/5-fluorocytosine requires the proliferation of the target cell. Moreover, 5-fluorouracil is used as a single agent in a limited number of cancers (gastrointestinal tract, for example). Potential problems have been associated with the use of 5-fluorouracil, however, such as the high doses necessary to achieve cytotoxicity and resistance [8,9].

Nitroreductase

Nitroreductase is a monomeric enzyme that converts non-toxic monofunctional alkylating agents to their difunctional forms. The products of these reactions are four orders of magnitude more toxic than the substrates [10]. *Escherichia coli* nitroreductase has been used to metabolize the prodrug 5-(aziridin-1-yl)-2,4-dinitrobenzamide to 5-(aziridin-1-yl)-4-(hydroxyamino)-2-nitrobenzamide, in the presence of cellular nicotinamide adenine dinucleotide, reduced form, or nicotinamide adenine dinucleotide phosphate, reduced form, acting as a reductant. The metabolized prodrug is then acetylated to 5-(aziridin-1-yl)-4-(acetylamino)-2-nitrobenzamide, which is capable of cross-linking cellular DNA, resulting in apoptosis. This system offers a large number of potential prodrugs that may be optimized. The prodrug currently used in conjunction with nitroreductase is CB1954 [11]. This system offers an advantage over HSV-TK and CD, in that it does not require cell proliferation to induce cell death.

Linamarase

Linamarase is a plant gene that hydrolyzes the cyanogenic glucoside substrate linamarin into glucose, acetone and cyanide. To date, a single study [12] has described the use of this enzyme for GPAT applications, but the originality of this system resides in the fact that the toxic component resulting from the conversion of the prodrug is a gas (cyanide) that can freely diffuse into the adjacent cells, inducing a strong bystander effect. Using this system, the eradication of very large intracerebral gliomas was reported in an animal model [12]. Furthermore, no appreciable toxic effects were observed. Further studies will be necessary to assess the real potential of this GPAT strategy.

Bystander effect

Antitumour gene therapy using GPAT strategies should, in theory, be limited to the cells that have been transduced with the suicide genes. Many investigators, however, have reported the induction of cell death in untransduced tumour cells. This phenomenon, referred to as the 'bystander effect', renders GPAT unexpectedly more efficient than initially predicted. For most of the suicide genes/prodrugs, the molecular mechanisms of this bystander effect have now been characterized, and can be divided into chemical and immunological bystander

effects. *In vivo*, both of these effects may be observed within the same experiment, but with different kinetics.

Chemical bystander effect

One of the first observations of this phenomenon was reported by Culver *et al* [13]. In these experiments, rat glioma cells were transduced with a replication-deficient retrovirus carrying the *HSV-TK* gene. Although only 10–70% of the tumour cells were transduced, very significant or complete tumour ablation was observed. Another study [14], with mixed tumour experiments, reported that 10% of HSV-TK-positive cells were sufficient in certain cases to observe tumour regression after treatment with ganciclovir [14]. A very elegant study [15] demonstrated that this bystander effect was the result of a metabolic cooperation, in which molecules of low molecular weight passed from one cell to another through gap junctions. In those experiments, the same cell line was transduced with either *HSV-TK* or the *LacZ* gene. When these cells were cocultured at low density, in which the majority of the cells were not in contact with each other, only the HSV-TK-expressing cells died. When the same experiment was repeated in conditions in which the cells were in contact, both cell lines were killed by the action of ganciclovir. These studies strongly suggested that phosphorylated ganciclovir may enter adjacent cells via the gap junction and cause cell death. Another study [16] attributed this bystander effect to the phagocytosis of apoptotic vesicles containing HSV-TK and metabolized ganciclovir, derived from dying cells.

Similar bystander effects were reported with other suicide genes/prodrugs, but the mechanism of toxicity varies among the different agents. For example, it has been shown [17] that 5-fluorouracil can diffuse to nearby tumour cells and pass through the cell membrane. A cell permeable metabolite is also responsible for the bystander effect observed with nitroreductase/CB1954 [18]. In the case of linamarase/linamarin, the cyanide gas produced freely diffuses from one cell to another [12].

Immunological bystander effect

The introduction of the *HSV-TK* gene into tumour cells, followed by the administration of ganciclovir *in vivo*, has also been demonstrated, in some cases, to induce the generation of a T-cell dependent antitumour immunity [19,20,21**]. For example, Kianmanesh *et al* [21**] demonstrated the existence of a 'distant' bystander effect; HSV-TK-positive or -negative malignant cells were seeded simultaneously in different rat liver lobes, in such a manner that there was no contact between the resulting tumours. After treatment of the rats with ganciclovir, both HSV-TK-positive and HSV-TK-negative tumours regressed and showed infiltration with macrophages and T lymphocytes. Protective immunity to the wild-type tumour was also induced when CD/5-fluorocytosine was used [22,23].

An important parameter that influences this immunological bystander effect is the way in which tumour cell death is induced. Apoptosis is usually associated with cell death in normal developmental processes [24], whereas cell death by a nonapoptotic pathway can be seen as a 'danger' signal (in viral-induced tissue lysis for example), and therefore is more likely to stimulate a immune response. HSV-TK/ganciclovir-mediated tumour killing can occur via apoptotic or nonapoptotic mechanisms, and experimentally the mechanism of HSV-TK-induced cell death can be diverted from apoptosis to nonapoptosis by manipulating intracellular levels of Bcl-2 [25**]. Using this system, Melcher *et al* [25**] demonstrated that HSV-TK-induced cell death by a nonapoptotic mechanism was associated with much higher immunogenicity than when tumour death was induced via an apoptotic pathway.

Intriguingly, a 'distant' bystander effect was reported [26] in a plasmacytoma model, when human tumour cells were transferred to severe combined immunodeficient mice. In these mice, a DNA-dependent protein kinase involved in immunoreceptor gene recombination is deficient. This genetic defect causes a complete absence of functional T as well as B lymphocytes. That study suggested that inflammatory cells or natural killer cells might participate in the distant bystander effect observed in immunocompetent animals.

Genetic prodrug activation therapy clinical trial for breast cancer

To date, only one phase I clinical trial using GPAT as a potential treatment for breast cancer has been described [27**]. The purpose of the trial was to test the safety and efficacy of tumour-specific expression of the CD gene driven by the *erbB-2* promoter. The *erbB-2* oncogene is overexpressed in 20% of breast carcinomas and is associated with reduced relapse-free and overall patient survival [28]. Twelve breast cancer patients received the transcriptionally targeted CD gene using direct, intratumoural injection of plasmid DNA, combined with systemic administration of the prodrug. The approach was shown to be safe and resulted in targeted expression of the CD gene in 90% of cases. Significant levels of expression of the suicide gene were detected and this expression was restricted to *erbB-2*-positive tumour cells. No significant, macroscopically observable tumor regression was reported, however. As a proof of principle, that study demonstrated the feasibility of exploiting transcriptional targeting to drive the expression of suicide genes.

Dominant-negative class of suicide genes

Many studies have been dedicated to the identification of the genetic defects that are associated with the formation and progression of tumours. Among these genetic abnormalities, activating mutations in the *ras* oncogenes contribute to around 30% of human malignancies [29]. In

laboratory experiments, constitutive activation of the Ras signalling pathway has been identified as essential for cellular transformation [30**]. Although Ras has clearly been implicated as an inducer of apoptosis in some cellular systems [31*], its role in carcinomas and myeloid cells is clearly to protect the transformed cells from apoptosis by inducing, in these cells, a strong survival signal [31*]. This survival pathway triggered by Ras activation has been partly characterized and encompasses phosphatidylinositol-3 kinase [32] and the protein serine/threonine kinase akt, also known as the protein kinase B [33]. These findings suggest that disruption of Ras function may be an effective approach in the treatment of carcinomas.

The elucidation of the Ras signalling pathway was made possible partly because of 'dominant-negative' proteins. These proteins are mutated versions of natural proteins and act as terminators of signalling pathways. For example, the dominant-negative Ras N116Y was created from the *v-H-ras* oncogene by substitution of tyrosine for asparagine at codon 116 [34]. This mutation leads to the destruction of the guanosine triphosphate-binding site and results in a catalytically inactive enzyme that can nevertheless bind to downstream elements of the signalling pathway. The dominant-negative will then compete with the normal Ras and may totally block the pathway. Using this approach, it was demonstrated that transfection of Ras N116Y to various human cell lines of malignant origin resulted in growth inhibition *in vitro* [35]. These results were expanded *in vivo*, when two oesophageal cell lines, containing a mutant Ras or a wild-type Ras, were used to seed tumours in nude mice [36*]. The animals then received a treatment that consisted of repeated injections of adenovirus carrying the dominant-negative Ras N116Y driven by the cytomegalovirus promoter. The treatment significantly reduced the growth of both cell lines *in vivo*, without any noticeable adverse effects. That study is, to our knowledge, unique thus far as it demonstrates the clinical potential of this strategy. Moreover, the elucidation of the complete Ras signalling pathway could give rise to a growing number of dominant-negative genes that could be used in conjunction to block the survival pathway.

Conclusion

Since the first experimental demonstration showing efficient killing of cells by a transfected gene encoding diphtheria toxin A chain, a variety of new genetic toxins have been designed and tested in preclinical studies. The first clinical trial of GPAT for breast cancer has just been completed, and demonstrated the safety and feasibility of the strategy. The long-term success of GPAT systems for clinical treatment of cancer will rely on the development of efficient and targeted gene therapy systems, however. This targeting can be achieved in two ways: targeted delivery, in which the gene medicine will be selectively delivered to the tumour cells; and transcriptional targeting,

which uses promoters that will be active only in tumour cells. Ideally, a combination of both targeting systems will be used.

As an alternative to GPAT, exploitation of the knowledge accumulated on mechanisms adopted by cancer cells to evade programmed cell death could produce genetic toxins such as dominant-negative mutants of the Ras signalling pathway that are more specific to cancer cells and do not need such sophisticated targeting systems. This type of approach is only just being tested, and safety issues remain to be addressed.

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