Improving enzymes for cancer gene therapy

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New techniques now make it feasible to tailor enzymes for cancer gene therapy. Novel enzymes with desired properties can be created and selected from vast libraries of mutants containing random substitutions within catalytic domains. In this review, we first consider genes for the ablation of tumors, namely, genes that have been mutated (or potentially can be mutated) to afford enhanced activation of prodrugs and increased sensitization of tumors to specific chemotherapeutic agents. We then consider genes that have been mutated to provide better protection of normal host tissues, such as bone marrow, against the toxicity of specific chemotherapeutic agents. Expression of the mutant enzyme could render sensitive tissues, such as bone marrow, more resistant to specific cytotoxic agents.

Keywords: directed evolution, gene therapy, myelosuppression, protein engineering, tumor ablation

A number of techniques, classified under the term "directed evolution," are currently being used to evolve enzymes in the test tube[1]. These methods allow exploration of large areas of sequence space to create active mutants, and then genetic selection or high-throughput screening can be used to identify mutant proteins that possess desired characteristics. One goal is to create mutant proteins with altered catalytic activities or substrate specificity. Many of these newly created enzymes have potential for cancer gene therapy.

Gene therapy for correction of genetic diseases such as sickle cell anemia involves substituting a mutant gene with its normal counterpart. However, this approach is unlikely to afford effective cancer therapy because human cancer cells contain mutations at multiple loci and have been postulated to exhibit a mutator phenotype that accelerates accumulation of further mutations[2]. Rather than address the many mutant genes, and variable combinations of mutant genes in cancer cells a logical approach is to introduce genes encoding prodrug metabolizing enzymes that have the potential to kill cancer cells[3–6]. Another approach involves protection of normal host tissues (e.g., bone marrow) against the toxicity that limits the dose escalation of many chemotherapeutic agents[6–9]. Chemoprotective genes can be transfected into bone marrow ex vivo, and only a small percentage of the cells need to be transfected. Cells expressing the drug-resistance gene should have a selective proliferative advantage in the presence of chemotherapeutic drugs, allowing these cells to overgrow unprotected marrow cells. Enrichment of resistant cells should enable higher doses of chemotherapy to be used, strengthening the probability of remission. The success of gene therapy might be increased by using mutated versions of enzymes for both tumor ablation and bone marrow protection.

Methods for evolving enzymes

A widely used technique for creating novel enzymes is site-directed mutagenesis. However, this approach is limited by our inadequate understanding of the effects of amino acid substitutions on structure and function. Moreover, analyzing multiple, rationally chosen substitutions, and combinations of multiple substitutions, by site-directed mutagenesis can be prohibitively arduous and time consuming. In contrast, directed evolution requires no previous knowledge of the changes necessary to achieve proteins with specific characteristics, because powerful selection or screening methods can be used to identify such proteins from libraries of millions of different mutants[1]. Some of the important methodologies that can be used to evolve enzymes for gene therapy have been described previously[1] and are summarized in Table 1. There are two subcategories of methods comprising directed evolution: first, those that create large libraries of mutants by nucleotide substitutions; and second, those that use in vitro homologous recombination (DNA shuffling).

With random oligonucleotide mutagenesis one can target and saturate multiple sites in a region of a plasmid-encoded gene. With chemical modification and error-prone PCR, one can scan larger gene segments to produce predominantly single mutations. DNA shuffling by random fragmentation or the staggered extension process affords more global diversity. All of these techniques yield a library of DNA inserts that can be ligated into an appropriate vector to create a plasmid library that can then be transformed into bacteria for genetic selection or screening. It must be emphasized that regardless of the technique used, the limiting factor in identifying novel enzymes is the ability to devise clever selections or screens for mutants possessing the features that are desired.

Prodrug metabolizing mutants for tumor ablation

Candidate genes for directed evolution and gene therapy are presented in Table 2. Those listed on the left have potential use for sensitizing tumors via prodrug metabolism. At present, herpes simplex virus type 1 (HSV) thymidine kinase (TK) is the most advanced of the candidates for tumor ablation and is therefore discussed here in further detail.

Thymidine kinase. Human TK catalyzes the phosphorylation of thymidine and a limited number of structurally related analogs. In contrast, HSV TK phosphorylates a variety of analogs, including ganciclovir and acyclovir. Ganciclovir and acyclovir lack a 3′-hydroxyl terminus and therefore act as chain terminators when incorporated into host DNA and/or viral DNA[10]. One should potentially be able to target cancer cells selectively by introducing HSV TK into tumors and then treating systemically with ganciclovir or acyclovir. This approach has been evaluated in animal models, where tumors regress following treatment with ganciclovir[11]. Although this method is in clinical trials, the immunosuppressive effects of the dosages needed for tumor regression currently limit efficacy.

The ability of HSV TK to sensitize tumors to ganciclovir or acyclovir could be enhanced by creating mutants that phosphorylate these analogs preferentially. Extensive random oligonucleotide mutagenesis of HSV TK has been carried out, yielding extremely promising mutants. A large library of mutants containing amino acid substitutions at the nucleoside binding site was created, and active clones were selected in an *Escherichia coli* strain deficient in TK[12]. *E. coli* harboring active mutant TKs were then screened for preferential killing at low doses of ganciclovir and acyclovir[13]. In the mutants sequenced, three to six amino acid changes were observed, suggesting that these variants could not have been rationally designed, and probably would never occur in nature. Some of these mutants were tested in hamster cells, and variants were identified that made the cells as much as 43-fold and 20-fold more sensitive to ganciclovir and acyclovir, respectively, compared with cells expressing wild-type HSV TK. One such mutant containing six amino acid changes near the active site has since been shown to have nanomolar IC_{50} values, compared with wild-type values of 30 μM and >100 μM for ganciclovir and acyclovir, respectively (Kokoris, M.S., Sabo, P., Adman, E., and Black, M.E., personal communication).

In companion studies, it was demonstrated using a mouse xenograft tumor model that a 10-fold lower dose of ganciclovir is required to impede the growth of tumors expressing this mutant, compared with tumors expressing wild-type TK. The mechanism for the increased sensitivity to these agents is thought to involve a 35-fold increase in the K_M for thymidine, resulting in reduced competition between the analogs and thymidine at the active site. The effectiveness in vivo is further enhanced due to the bystander effect, where cells not expressing the mutant TK apparently can be killed when they are adjacent to transduced cells[14]. A role for gap junctions in this mechanism has been proposed[15].

HSV TK mutants with altered activity toward azidothymidine (AZT) have also been developed using directed evolution. Use of DNA family shuffling of different species of HSV TK allowed mutants with multiple amino acid substitutions to be uncovered; these mutants sensitized bacteria to significantly lower concentrations of AZT than amounts required to kill cells expressing wild-type HSV TK to the same extent (Christians, F.C., Scapozza, L., Crameri, A., Golkers, G., and Stemmer, W.P.C., personal communication). The increased sensitivity has been attributed to a lower K_M for AZT in the mutant, and a loss of specificity for thymidine. This mutant offers the possibility of using the chain terminator, AZT, for cancer gene therapy.

Drug-resistance mutants for protecting bone marrow

The right side of Table 2 summarizes some of the gene products that in principle might confer increased cellular resistance to specific cancer chemotherapeutic agents. Some of the candidates shown are known to be expressed in low or inadequate levels in bone marrow precursor cells and therefore could limit cell survival. The potential for bone marrow protection has been strengthened by gene transfer experiments in relevant mammalian cells, such as hematopoietic stem cells[9], and, in some cases, by success in animals[16,17]. Human cDNAs for O^6-alkylguanine-DNA alkyltransferase (AGT), thymidylate synthase (TS), dihydrofolate reductase (DHFR), and glutathione-S transferase (GST) are currently being evaluated, as described below.

O^6-Alkylguanine-DNA alkyltransferase. AGT removes alkyl adducts from the O^6-position of guanine in DNA by a mechanism that autoinactivates the protein[18]. O^6-alkylguanine is a major mutagenic and cytotoxic lesion found in DNA following exposure to a variety of alkylating agents, including chemotherapeutic *N*-nitroso compounds such as 1,3, bis(2-chloroethyl)1-nitrosourea (BCNU)[18]. When BCNU and other alkylating agents are used clinically, the limiting factor in dose escalation is usually myelosuppression[9]. Wild-type human AGT has been overexpressed in the bone marrow of mice using retrovirus-mediated gene transfer, thus protecting AGT-expressing cells from the cytotoxicity of BCNU[16].

Mutant AGTs that display enhanced activity have been created by directed evolution, and isolated by selection in *E. coli* that lack alkyltransferase. One mutant, Val139→Phe, with a single amino acid substitution near the active site cysteine (Cys 145), rendered *E. coli* more resistant to killing and mutagenicity following treatment with the simple methylating agent, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) than wild-type AGT[19]. In another study, AGT was shuffled against the sequences of all known mammalian alkyltransferases, and mutants were identified in bacteria by using selection for MNNG resistance. A mutant with seven amino acid substitutions, all distant from the active site, was found to be the most resistant clone, again affording better protection than wild-type AGT (Christians, F.C. and Stemmer, W.P.C., personal communication).

O^6-Benzylguanine (BG), a potent competitive inhibitor of AGT, is currently in clinical trials for improving cancer chemotherapy[18]. The hypothesis is that BG depletes tumor cells of AGT, thereby increasing the cytotoxicity of alkylating agents. However, BG is not tumor specific and presumably further reduces the already low levels of AGT activity in bone marrow. Thus, mutant AGTs that are resistant to BG, yet retain the ability to remove alkyl adducts from

Table 1. Summary of directed evolution techniques that can be used to create novel enzymes.

Nucleotide substitutions	In vitro recombination (DNA shuffling)
Random oligonucleotide mutagenesis[48]	Random DNA fragmentation[51]
Chemical mutagenesis[49]	Staggered extension process[52]
Error-prone PCR[50]	

Table 2. Summary of enzymes that could be used for gene therapy and their applications.

Tumor ablation		Protection of bone marrow	
Enzyme	**Prodrug**	**Enzyme**	**Therapeutic agent**
HSV TK	GCV, ACV[1,5,6]	AGT	alkylating agents, BG[1,6,9]
P450	procarbazine, dacarbazine, CPA, IFA[5,6,53–55]	TS	5-FU, folate analogs[8,9]
		DHFR	MTX, TMTX[8,9]
		GST	mechlorethamine[1]
		Pgp	anthracyclines, vinca alkaloids, taxol[6,8,9]
ThdPase	dFUr[5]		
CK	ara-C[5]	AAG	alkylating agents[56]
CD	5-FC[5,6]	Topo I	camptothecin[8]
E. coli gpt	6TX[5]	Topo II	anthracyclines[8]
E. coli nitroreductase	CB 1954[5,6]	ADH	cyclophosphamide[9]
C. acetobutylicum electron transport system	metronidazole[5]	RR	hydroxyurea[8]
		metallothionein	cisplatin, chlorambucil[9]
		tubulin	vinca alkaloids, taxol[8]

Abbreviations not defined in text: GCV, ganciclovir; ACV, acyclovir; CPA, cyclophosphamide; IFA, ifosfamide; ThdPase, thymidine phosphorylase; dFUr, 5'-deoxy-5-fluorouridine; CK, deoxycytidine kinase; ara-C, cytosine arabinoside; CD, cytosine deaminase; 5-FC, 5-fluorocytosine; gpt, guanine phosphoribosyl transferase; 6TX, 6-thioxanthine; CB 1954, [5(-aziridine-1-yl)-2,4-dinitrobenzamide; 5-FU, 5-fluorouracil; MTX, methotrexate; TMTX, trimetrexate; Pgp, P-gylcoprotein; AAG, 3-methyladenine DNA glycosylase; Topo I, topoisomerase I; Topo II, topoisomerase II; ADH, aldehyde dehydrogenase; RR, ribonucleotide reductase.

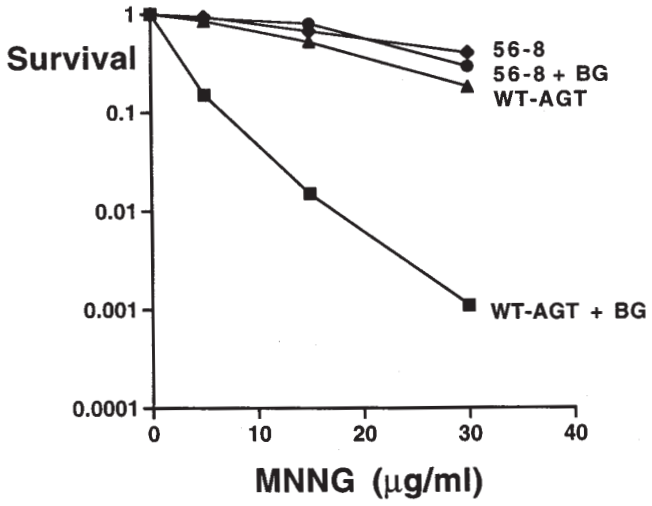

Figure 1. Survival of alkyltransferase-deficient bacteria expressing wild-type or mutant 56-8 (Cys150→Tyr, Ser152→Arg, Ala154→Ser, Val155→Gly, Asn157→Thr, Val164→Met, Glu166→Gln, Ala170→Thr) AGT in the presence of MNNG ± BG (100 μM). In this experiment, cells were treated with graded doses of MNNG for 10 min at 37°C, and spread on plates for colony counting to determine survival.

DNA, could be highly advantageous for protecting bone marrow. Such mutants were originally identified by site-directed mutagenesis, the presumptive mechanism of resistance being a restructuring of the active site to prevent entry of the larger benzyl group. However, these mutants, most of which contain single substitutions, offer only partial resistance to BG in vitro (e.g., Gly156→Ala)[18]. In addition, some of these mutants provide bacteria with less protection against MNNG alone (Encell, L.P. and Loeb, L.A., unpublished data) and had shorter intracellular half-lives than wild-type AGT in mammalian cells (Davis, B.M., Roth, J.C., Liu, L., Freibert, S.E., Schupp, J.E., Neumann, A. et al. personal communication).

Random oligonucleotide mutagenesis has since been used to select for AGT mutants that are resistant to BG[20–22]. Mutant 56-8 (Cys150→Tyr, Ser152→Arg, Ala154→Ser, Val155→Gly, Asn157→Thr, Val164→Met, Glu166→Gln, Ala170→Thr) is particularly interesting because it contains eight amino acid substitutions within a 21 amino acid stretch near the active site. Although all of the mutations are probably not required for the change in substrate specificity, it is nevertheless remarkable that a protein can tolerate this many mutations and still be functional. Figure 1 illustrates the capacity of 56-8 to protect alkyltransferase-deficient bacteria against killing by MNNG (Encell, L.P. and Loeb, L.A., unpublished data).

Another interesting variant is the single mutant, Pro140→Lys[22]. In addition to being highly resistant to BG in *E. coli*, this mutant was resistant to BG in Chinese hamster ovary cells[23] and hematopoietic cells (Davis, B.M., Roth, J.C., Liu, L., Freibert, S.E., Schupp, J.E., Neumann, A. et al. personal communication), and protected the cells against BCNU as well as wild-type AGT.

Some of the earlier discovered BG-resistant mutants have also been examined in mammalian cells, as well as in mice[24,25]. Both Gly156→Ala and a double mutant (Pro140→Ala, Gly156→Ala) have been introduced to mouse bone marrow ex vivo by using retroviral constructs. Gly156→Ala protected against lethality in mice treated with BCNU and BG, whereas the double mutant protected bone marrow against the toxic and clastogenic effects of the alkylating agent, temozolomide, in combination with BG. Gly156→Ala is currently being evaluated for bone marrow protection in patients receiving combination therapy including BCNU and BG. However, some of the more recently established BG-resistant mutants such as Pro140→Lys might be more effective due to greater resistance to BG.

Thymidylate synthase. TS catalyzes the methylation of dUMP to dTMP by using methylenetetrahydrofolate as a methyl donor. After further phosphorylation, dTMP is incorporated into DNA. The production of dTMP by this de novo pathway is required for DNA synthesis and cell survival, and TS activity is highest in rapidly dividing cells[26]. For these reasons, human TS has been an important target for the design of chemotherapeutic agents[27,28]. First used over 40 years ago, 5-fluorouracil, an inhibitor of TS, remains a mainstay for the treatment of breast and gastrointestinal tumors. As in the case of chemotherapeutic alkylating agents, myelosuppression often limits the maximum tolerated dose of 5-fluorouracil[29]. In order to increase tumor ablation efficacy, stable and specific folate-based inhibitors have been designed based on the TS crystal structure. One compound, Tomudex (ZD 1694; Zeneca Pharmaceuticals, UK), has been recently approved for treatment of advanced colorectal cancer.

Over 10 years ago, a 5-fluorouracil–resistant human colonic cell line was found that harbored the TS variant, Tyr33→His, raising the possibility that mutants could be used to protect the bone marrow of patients receiving 5-fluorouracil therapy[30]. Recently, drug-resistant TS enzymes have been created by chemical mutagenesis of human sarcoma cells, followed by selection with the folate analog, Thymitaq (AG337; Agouron Pharmaceuticals, La Jolla, CA)[31]. The most striking mutants increased the IC_{50} of mouse TS-deficient cells up to 40-fold against Thymitaq and 96-fold against 5-fluorodeoxyuridine. Many alterations were found in the highly conserved arginine-50 loop, which is believed to form an important bridge linking the substrate, cofactor, and enzyme C terminus. This work suggests that not only is this loop central to binding of the inhibitors Thymitaq and 5-fluorodeoxyuridylate, but it can be altered to allow a specific change in inhibitor binding while retaining nearly unaltered binding of the normal substrates.

Targeted evolution of a region of human TS near the catalytic cysteine (position 195) has also produced novel TS 5-fluorodeoxyuridine-resistant mutants[32]. A plasmid-encoded library of TS mutants was transfected into *E. coli* lacking the *ThyA* (TS) gene. Clones that were able to survive in the presence of increasing doses of 5-fluorodeoxyuridine were sequenced, and it was discovered that altering residues 197–199 is sufficient to provide increased 5-fluorodeoxyuridine resistance. Kinetic analysis of a triple mutant (Ala197→Val, Leu198→Ile, Cys199→Phe) demonstrated a 20-fold increase in K_d for 5-fluorodeoxyuridylate, with retention of near wild-type catalytic efficiency and affinities for dUMP and methylenetetrahydrofolate.

As our knowledge of TS structure increases, it is becoming possible to predict alterations that may result in drug resistance. Recently, creation of 14 site-directed mutants in the folate binding site has yielded novel mutants resistant to Thymitaq, 5-fluorodeoxyuridine, and Tomudex[33]. Although the crystal structure of human TS has been solved, structures of the mutants bound to either a pyrimidine-based inhibitor and the methylenetetrahydrofolate cofactor or dUMP and a folate analog will be crucial in furthering our understanding of the mechanisms by which individual residue alterations lead to drug resistance.

Dihydrofolate reductase. DHFR functions to regenerate tetrahydrofolate from dihydrofolate, a product of TS. Inhibition of DHFR activity by methotrexate results in decreased thymidylate and purine biosynthesis, and cell death. Mutant DHFRs that confer resistance to methotrexate and other folate analogs have been reported by several laboratories[34–36]. Early studies identified Leu22→Arg and Phe31→Ser substitutions that confer resistance to

methotrexate in a human colon carcinoma cell line. Unlike Phe31→Ser, Leu22→Arg exhibits extremely low catalytic activity[37]. In addition, saturation mutagenesis has identified Leu22→Tyr and Leu22→Trp as drug-resistant, yet less catalytically impaired, enzymes[38]. Double mutants containing substitutions at residues 22 and 31 have been created and demonstrate greater drug resistance compared with their single mutant progenitors[39].

Both microinjection and retroviral infection of mouse bone marrow cells with an altered DHFR cDNA followed by transplantation to normal irradiated mice has been shown to protect the mice from lethal doses of methotrexate[40–42]. Recently, an elegant set of experiments demonstrated proof of the concept that increased drug dosage after transplantation with bone marrow expressing a variant gene will lead to improved curability of a tumor. Mice transplanted with murine mammary adenocarcinoma were treated with high doses of cyclophosphamide and subsequently transplanted with bone marrow transduced with a mutant human DHFR cDNA (Phe31→Ser)[43]. Forty-four percent of these mice had no demonstrable tumor on day 52, whereas mice transplanted with unmodified bone marrow all died of methotrexate toxicity, and mice untreated by methotrexate succumbed to tumor regrowth. These results are promising in that not only is a higher than conventional dose of methotrexate tolerated by the mice, but this increased dose is associated with improved tumor ablation.

As newer folate analogs are developed, mutants of the corresponding resistance gene can be created for gene therapy. Trimetrexate is one such analog that is active against certain methotrexate-resistant tumors. Through screening of methotrexate-resistant DHFR variants, Leu22→Tyr was found to yield almost 100-fold increased resistance to trimetrexate relative to controls[44]. Subsequently, mice reconstituted with Leu22→Tyr bone marrow cells were found to be protected from trimetrexate-induced neutropenia and reticulopenia.

Coexpression of a metabolically related gene can potentiate resistance to methotrexate. A retroviral vector containing both the HSV TK gene and DHFR Phe31→Ser cDNA has been shown to dramatically increase methotrexate resistance compared with the DHFR Phe31→Ser cDNA construct alone, due to increased salvage of thymidine by HSV TK[45]. Similar coexpression of the *E. coli* xanthine-guanine phosphoribosyltransferase gene with the Phe31→Ser DHFR gene yielded comparable effects, due to increased salvage of purines[46]. Coexpression of mutants of different genes that confer resistance to specific agents could be used to design novel combination drug therapies.

Glutathione-*S*-transferase. Mammalian cells use GST to detoxify a diverse group of compounds, including chemotherapeutic nitrogen mustards such as mechlorethamine. In order to obtain active GSTs with altered substrate specificity, random substitutions have been introduced into the active site[47]. Bacteria harboring randomly substituted GST mutants were exposed to multiple rounds of increasing concentrations of mechlorethamine. GST variants were identified that exhibited up to a 10-fold increase in resistance to mechlorethamine cytotoxicity relative to wild-type GST. These mutants are currently being evaluated in gene transfer experiments using mammalian cells. Interestingly, the mutant that conferred the greatest resistance contained three amino acid substitutions, all of which were required concomitantly for enhanced survival. These studies suggest that GST could be further evolved to detoxify a variety of drugs by simply using the drug of interest in the selection assay.

Perspectives

Cancer gene therapy holds enormous promise. Yet, as is also true for its innovative targeting counterparts (e.g., antisense technology or peptide-guided drug delivery), many technical obstacles remain to be surmounted. Current goals include identifying or creating vectors that afford tissue and tumor specificity, enhanced efficiency of transduction, and conditional expression in human cells. There are potential clinical problems as well. For example, although gene therapy may protect marrow cells from the cytotoxicity of chemotherapeutic agents, surviving cells may sustain heightened premutagenic DNA damage that increases the frequency of subsequent leukemias.

Novel gene products add additional options to a gene therapy strategy; yet, there are potential problems specific to mutant enzymes. For example, expression of mutant proteins that are immunogenic may render normal cells, as well as tumor cells, subject to destruction by the immune system. In the absence of tumor-specific vectors, it may be necessary to evolve mutant enzymes that combine desired catalytic properties and low immunogenicity. With both promise and problems in view, it is apparent that directed evolution techniques are only beginning to play a role in cancer gene therapy and that as these techniques advance, their contribution to gene therapy can only grow.

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