expert reviews in molecular medicine

Genetic manipulation of drug sensitivity in haematopoietic cells

Thomas Southgate and Leslie J. Fairbairn

The haematopoietic system can be manipulated genetically to increase either its resistance to drugs or its sensitivity to certain agents. Gene transfer and expression of specific drug-resistance factors might protect haematopoietic function during antitumour chemotherapy, or allow enrichment of gene-modified cells in vivo. By contrast, gene transfer of a prodrug activator, to confer sensitivity to otherwise nontoxic prodrugs, might allow deletion of engrafted cells in the event of an adverse effect such as graft-versus-host disease or the induction of a neoplasm. In addition, expression of a prodrug activator in tumour-infiltrating haematopoietic cells could provide a means of specifically activating a cytotoxic agent within a tumour mass.

The genetic manipulation of drug sensitivity in the haematopoietic system can be carried out in order to achieve different goals. First, the aim might be to increase drug resistance to protect haematopoietic stem cells (HSCs) and progenitor cells from cytotoxicity during antitumour chemotherapy. This has now been extended to encompass efforts to achieve in vivo selection of gene-modified cells for treatment of other acquired and inherited diseases. Second, the aim might be to sensitise HSCs to cytotoxicity in order to achieve selective killing of gene-modified cells, through expression of a product that activates a noncytotoxic prodrug to a toxic metabolite. This has been investigated as a means to overcome potential complications of gene therapy and allogeneic bone marrow transplantation, and also as a means of using haematopoietic cells as vectors to deliver cytotoxic metabolites to tumours.

Engineering drug resistance

Genetic chemoprotection

Cytotoxic chemotherapy is a mainstay of many anticancer treatments. A wide range of drugs have been developed that show varying degrees of efficacy against various tumours following systemic administration. However, such drugs

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are not tumour specific and also kill normal cells, leading to unwanted toxicities in normal tissues. Such toxicities can become dose-limiting, leading to modifications in treatment and to insufficient tumour reduction. Thus, changing the balance between tumour- and normal-cell killing presents an attractive strategy for improving the effectiveness of currently used chemotherapeutic agents.

Normal tissues most at risk from collateral toxicity are those with a high proliferative index, such as bone marrow and gut epithelium, although there are several agent-specific toxicities in many other organs (Ref. 1). In the haematopoietic system, chemotherapeutic damage and killing within the stem and progenitor cell compartments can lead to profound myelosuppression, with neutropaenia and thrombocytopaenia, leading to increased risks of infectious complications and bleeding, respectively (Refs 2, 3). Supportive care, using haematopoietic growth factors or infusions of mobilised peripheral blood stem cells, can be used to enhance haematopoietic recovery after chemotherapy. However, the resulting haematopoietic system is still sensitive to further administrations of chemotherapeutic agents (Refs 4, 5).

Thus, several groups have developed vectors and strategies to confer genetic chemoprotection upon bone marrow stem and progenitor cells. To do this, gene transfer is used to achieve high-level expression of specific drug-resistance factors in bone marrow. It is hoped that this strategy will provide a haematopoietic system that is refractory to the cytotoxic effects (and potentially other effects) of chemotherapeutics. Various drug-resistance mechanisms can be employed (Table 1) and, through these, protection against a wide range of clinically useful agents might be achieved. For the purposes of this review, we concentrate on two drug-resistance factors: MDR-1 (multiple drug resistance 1; also known as P-glycoprotein or gp170) and MGMT (O^6-methylguanine-DNA-methyltransferase; variously called ATase or AGT).

MDR-1

MDR-1 is a member of the ATPase-binding cassette (ABC) family of proteins, which also includes multidrug resistance proteins (MRPs) and ABCG2 (Ref. 6). MDR-1 is an ATP-dependent membrane protein that acts as an efflux pump, actively exporting xenobiotics from cells. Expression of MDR-1 confers resistance to a wide

Table 1. Common genetic chemo- and radioprotection strategies

Resistance mechanism	Proteins responsible	Agents against which resistance conferred	Refs
ABC transporters	MDR-1, MRP-1, ABCG2	Anthracyclins (e.g. daunorubicin), vinca alkaloids (e.g. vincristine), epipodophylotoxins (e.g. etoposide)	6
DNA repair functions	MGMT, various glycosylases	Alkylating agents (e.g. BCNU, temozolomide)	97, 162
Drug detoxification	Aldehyde dehydrogenase	Oxazaphosphorines (e.g. cyclophophamide)	163
	Glutathione *S*-transferase	Alkylating agents, anthracyclins	164
Redox	Superoxide dismutase 2	Radiation	165
Antimetabolite resistance	Mutant dihydrofolate reductase	Methotrexate	103
	Thymidylate synthase	5-Fluorouracil	166
	Cytidine deaminase	Ara-C, gemcitibine	167

Abbreviations: ABCG2, ATPase-binding cassette G2 protein; Ara-C, cytosine arabinoside; BCNU, carmustine; MDR-1, multiple drug resistance 1; MGMT, O^6-methylguanine-DNA-methyltransferase; MRP-1, multidrug resistance protein 1.

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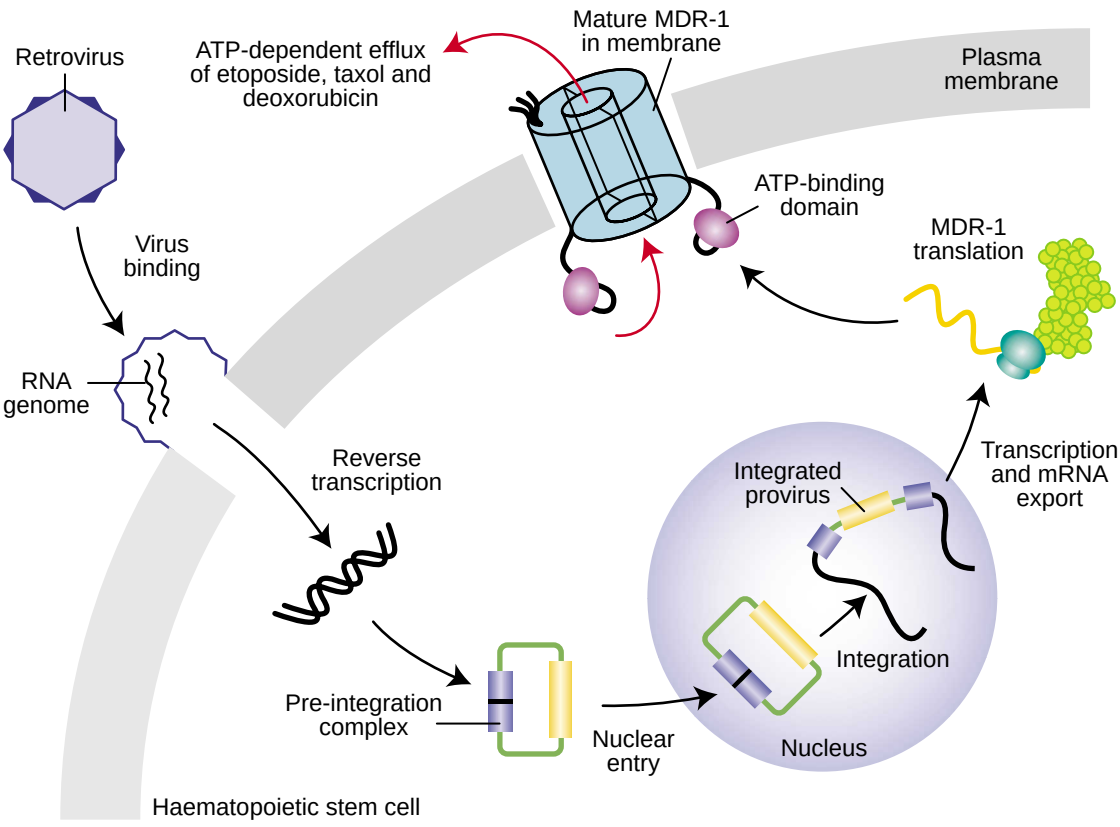
range of chemotherapeutic agents, including podophylotoxins, anthracyclins, vinca alkaloids, actinomycin D and taxol (Ref. 7). Dysregulated expression of MDR-1 is a major determinant of tumour-cell resistance to therapy, and MDR-1 expression has been correlated to poor response and outcome in some studies and some tumours (Refs 8, 9).

Early indications that MDR-1 expression could help to overcome collateral haematopoietic toxicity came from experiments in transgenic mice in which MDR-1 was ectopically expressed in bone marrow (Ref. 10). Such animals showed resistance of haematopoiesis to leukopaenia following treatment with either of the antitumour drugs daunomycin or taxol, which was reversed by the MDR-1 inhibitor verapamil (Ref. 11). Moreover, transplantation of bone marrow from *MDR-1*-transgenic mice to nontransgenic animals conferred drug-resistant haematopoiesis to these recipients (Ref. 12). These studies established the principle that MDR-1 overexpression in bone marrow could provide a chemoprotective effect.



Retroviral transfer of *MDR-1* confers drug resistance

Figure 1. Retroviral transfer of *MDR-1* confers drug resistance. The retroviral vector encoding MDR-1 (multiple drug resistance 1) binds to its cognate receptor on the surface of a target cell and undergoes fusion with the plasma membrane. The RNA genome of the retrovirus is reverse transcribed within the cytoplasm of the target cell to provide a double-stranded DNA copy, which then forms a pre-integration complex. This interacts with genomic DNA when the nuclear envelope breaks down at mitosis, and the virally encoded integrase facilitates integration of the proviral genome into the nuclear DNA. The integrated provirus is transcribed by nuclear transcription factors and the mRNA exported to the cytoplasm for translation. The mature MDR-1 protein locates to the plasma membrane where it functions to provide ATP-dependent efflux of chemotherapeutic drugs such as etoposide, taxol and doxorubicin.

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On the basis of these and in vitro gene-transfer studies (Refs 13, 14, 15, 16), retroviral *MDR-1*-gene-transfer experiments in murine bone marrow were undertaken by several groups (Fig. 1). Again, clear evidence of a myeloprotective effect was seen in animals transplanted with retrovirally transduced bone marrow (Refs 16, 17, 18, 19). Moreover, in mice carrying tumours, protection of haematopoiesis by *MDR-1* gene transfer facilitated dose escalation and led to improved survival of animals (Ref. 20). Further evidence of transduction and protection of human $CD34^+$ cells in vitro was obtained (Refs 19, 21) prior to initiation of clinical trials of MDR-1-based genetic chemoprotection.

The trials conducted to date have mainly been characterised by low frequencies of gene transfer to repopulating cells and transient levels of *MDR-1*$^+$ cells (Refs 22, 23, 24, 25, 26, 27). This most probably reflects poor transduction of cells with true long-term-repopulating capacity, which in turn reflects the state of the art in human HSC transduction at the time these trials were instigated. Furthermore, it is likely that the retroviral vectors employed for these studies were less than optimal for expression in primitive cells. Not withstanding this, in a few patients, MDR-1^+ cells have either appeared or increased in number post-chemotherapy, although the levels remained fairly low (Ref. 27). Although the number of patients showing this effect are too low to conclude positively that a selective event occurred (as opposed to clonal fluctuations in stem cell usage), these data still give a sense that MDR-1-based chemoprotection might be of value if attention is given to the technical aspects of gene transfer, expression and drug selection.

Many of the technical requirements for improved gene transfer and expression of MDR-1 in bone marrow are generic and will also be required for other gene therapy applications. The identification of cytokine conditions that support repopulating cells during the transduction process (Refs 28, 29) and the use of a fibronectin fragment to improve transduction (Refs 30, 31) have been reported. Such conditions were used in a further clinical trial of MDR-1-based chemoprotection. In this case, higher levels of gene marking were seen in some patients at up to one year post-transplant, and there was also some evidence for in vivo selection (Ref. 32). Improved retroviral vectors based on the spleen focus-forming virus (SFFV) long-terminal repeat (LTR) promise higher and more-sustained levels of expression in HSCs and progenitor cells (Refs 33, 34), whereas pseudotyping vectors with the envelope of the gibbon ape leukaemia virus (GALV) can facilitate improved gene transfer to repopulating stem cells of humans and other primates (Refs 29, 31, 35, 36, 37).

Following these technical improvements, transfer and expression of *MDR-1* has now been demonstrated in human cells capable of engrafting in the immunodeficient nonobese diabetic (NOD)/severe combined immune deficiency (SCID) mouse transplantation model (Refs 38, 39). Moreover, protection and selection of MDR-1-expressing cells has been demonstrated in this model. Further improvements have included modification of cryptic splice acceptor sites in the *MDR-1* cDNA that have previously led to reduced expression of MDR-1 protein, and the use of post-transcriptional regulatory elements (Ref. 40). Such advances, along with the demonstration of gene transfer, expression and protection in large-animal models (Ref. 41), should lead to further clinical assessment of MDR-1-based chemoprotection once previously raised concerns over an MDR-1-derived toxicity (Ref. 42) (see below) have been fully addressed.

MGMT

MGMT specifically repairs O^6-alkylguanine (O^6-alkG) adducts in DNA (Ref. 43) .These adducts have been shown to be cytotoxic, mutagenic, clastogenic (causing chromosome breaks) and carcinogenic (Ref. 44). Agents that induce O^6-alkG in cellular DNA include several clinically useful antitumour agents such as chloroethylnitrosoureas [e.g. biodegradable carmustine (BCNU)] and O^6-methylating agents [e.g. dacarbazine (DTIC) and temozolomide] (Ref. 43). Overexpression of MGMT is associated with tumour-cell resistance to O^6-alkylating agents, with the levels of MGMT activity inversely correlated with tumour xenograft responses (Refs 45, 46).

Early experiments established that ectopic expression of a bacterial analogue of MGMT in otherwise sensitive normal cells conferred resistance to the cytotoxic, clastogenic and mutagenic effects of a range of O^6-alkylating agents in vitro (Refs 47, 48). Moreover, overexpression of MGMT in tissues of transgenic mice led to protection of these animals against acute cytotoxicity and carcinogenesis following

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their exposure to nitrosoureas (Refs 49, 50). These studies provided the impetus for gene-transfer experiments in bone marrow (Refs 51, 52, 53), and several groups showed that transfer and expression of wild-type MGMT following transplantation led to protection of the haematopoietic system of recipient animals (Refs 54, 55).

MGMT detoxifies O^6-alkG by transferring the lesion to a cysteine in the MGMT active site (Ref. 56). This is a covalent and irreversible reaction, and leads to inactivation of MGMT. The protein is then ubiquinated and degraded (Ref. 57). This has led to the development of clinical strategies aimed at ablating tumour-cell MGMT prior to treatment with antitumour agents. Early attempts to sensitise tumours in this way centred on the use of O^6-methylating agents to ablate tumour MGMT prior to treatment with a chloroethylating agent (Refs 58, 59). However, it soon became apparent that such an approach increased collateral toxicity in bone marrow to an unacceptable level and this was abandoned. Current strategies use small-molecule mimics of O^6-alkG in DNA as pseudosubstrates for MGMT. These agents, of which O^6-benzylguanine (O^6-beG) and O^6-bromothenylguanine (PaTrin2) are in clinical trial, react with the active site of MGMT and very effectively ablate tumour resistance (Refs 60, 61) (Fig. 2). However, none of the pseudosubstrates acts specifically on tumour tissue, and collateral toxicity to haematopoietic cells was demonstrated in human haematopoietic progenitors in vitro (Refs 62, 63) and mouse bone marrow in vivo (Ref. 64). This predicted increased toxicity has now been confirmed in Phase I and II clinical trials of MGMT inactivators, where increased myelosuppression was observed that resulted in a reduction of the maximum tolerated dose of O^6-alkylating agent (Refs 65, 66).

Studies in bacteria have indicated that the two *Escherichia coli* analogues of MGMT (Ada and Ogt) exhibit resistance to inactivation (Ref. 67). Moreover, transfer and expression of the *E. coli ada* gene could confer inactivator-insensitive protection to bone marrow cells (Ref. 68). On the basis of such observations, several mutant versions of the human MGMT have been produced, with varying degrees of resistance to O^6-beG (Refs 69, 70, 71, 72) (Table 2) and PaTrin2 (L. Fairbairn, unpublished). The use of mutant MGMT in gene-transfer experiments has quickly established its ability to protect murine and human haematopoiesis against the toxicity and clastogenicity of combinations of O^6-alkylating agent and inactivator (Refs 73, 74, 75, 76) (Fig. 2). Further studies have subsequently demonstrated that protection of bone marrow in this way led to increased survival of animals that were challenged with chemotherapy and to an increased therapeutic index against tumour xenografts (Refs 75, 77, 78). Clinical trials of mutant MGMT-based chemoprotection, in order to test the potential of this strategy for protection and selection, are eagerly awaited.

Dual expression

Only a few antitumour regimens make use of a single cytotoxic agent (or class of agents). Instead, chemotherapy is often administered in a multi-agent fashion with a view to potentially overcoming tumour-cell resistance to individual drugs (Ref. 79). Thus, the collateral cytotoxicity seen on treatment represents the combinatorial toxicity of multiple agents. Although protection against one component of a multi-agent regimen might reasonably be expected to reduce cytotoxic side effects to some extent, protection of sensitive tissues against all toxicities that result from the various agents used would clearly be preferable. To this end, several groups have explored the potential of vectors that coexpress more than one drug-resistance function. Thus, *MDR-1* has been combined with *MGMT* in a retroviral vector, and data showing in vitro protection of cells against

Figure 2. MGMT activity and cellular sensitivity to O^6-alkylating agents. *(Legend; see next page for figure.)* (a) Exposure of tumour cells to an O^6-alkylating agent such as temozolomide leads to alkylation of DNA at the O^6-position of guanine. If the tumour cell expresses little or no MGMT (O^6-methylguanine-DNA-methyltransferase), drug exposure can lead to cell death; however, if MGMT is expressed to a sufficient level the alkyl group is transferred to the active site of MGMT, in a stoichiometric and autoinactivating manner, resulting in DNA repair and tumour-cell resistance. Haematopoietic stem cells (HSCs) express little or no MGMT and are particularly sensitive to temozolomide. (b) Addition of an MGMT pseudosubstrate, such as O^6-benzylguanine, leads to inactivation of MGMT and sensitisation of tumour cells to the cytotoxicity of temozolomide. At the same time, retroviral transduction of HSC with a mutant (O^6-benzylguanine-insensitive) MGMT leads to inactivator-insensitive protection against the cytotoxicity of temozolomide.

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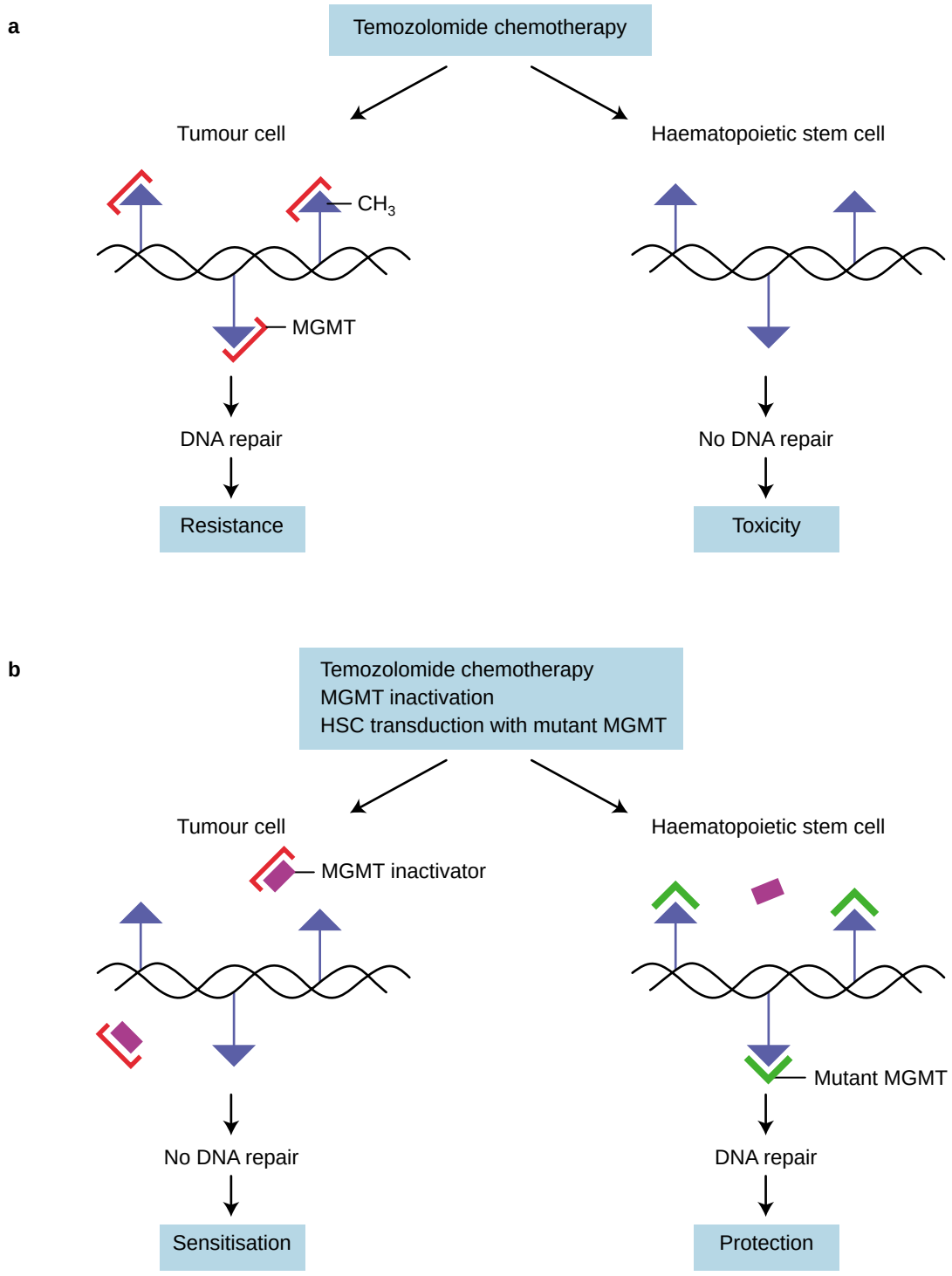

MGMT activity and cellular sensitivity to O^6-alkylating agents

Figure 2. MGMT activity and cellular sensitivity to O^6-alkylating agents. *(See previous page for legend.)*

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Table 2. Inactivator-resistant human MGMT mutants[a]

MGMT mutant	IC_{50} O^6-beG (μM)	Relative resistance (-fold)	Refs
Wild type	0.2	1.0	69, 73
P140A	2.5–5.0	12.5–25.0	69, 73
G156A	15–60	75–300	69, 70
P140K	>1200	>6000	70, 72
P140A/G156A	>500	>2500	73

[a] The table shows the effect of specific point mutations on the sensitivity of human MGMT to inactivation by O^6-beG, expressed as the concentration of inactivator required to reduce MGMT activity by 50% (IC_{50}). Relative resistance (-fold), compared with wild-type MGMT, is also shown.
Abbreviations: MGMT, O^6-methylguanine-DNA-methyltransferase; O^6-beG, O^6-benzylguanine.

combinations of agents have been obtained (Refs 80, 81, 82). Similar studies have combined MDR-1 with either a mutant dihydrofolate reductase (conferring resistance to methotrexate) (Ref. 83) or aldehyde dehydrogenase (conferring resistance to cyclophosphamide) (Ref. 84).

Where dual expression is required, it will probably be important to achieve high-level expression of both resistance factors in order to maximise the protective effect of gene transfer. Most vectors used to date have utilised an internal ribosome entry site (IRES) that permits binding of ribosomes midway through an mRNA transcript and initiation of translation of a second cistron independently of cap-driven translation of the upstream coding sequence (Refs 85, 86). Since both trangenes are expressed from a single mRNA molecule, an advantage of using an IRES is that expression of one gene product is almost invariably associated with expression of the other, thus assuring coexpression. One disadvantage, however, stems from the observation that the level of translation of the downstream coding sequence can be as low as 10% of that from the upstream cistron (Ref. 87). Some groups have overcome this by producing fusions of two resistance functions (e.g. a chimaeric gene encoding mutant dihydrofolate reductase fused to thymidylate synthase, to confer resistance to both methotrexate and 5-fluorouracil) (Refs 88, 89). However, this is a tenable strategy only where both resistance functions perform their protective role within the same cellular compartment (e.g. the cytoplasm). An alternative approach, which might allow both high-level production of more than one protein and appropriate cellular localisation, makes use of the self-processing 2A moiety of the foot and mouth disease virus (Ref. 90). Incorporation of this as part of a fusion protein between an upstream and downstream coding sequence results in cotranslational processing of the nascent polypeptide chain, allowing release of the upstream protein from the ribosome yet continued synthesis of the downstream protein (Refs 89, 91). The use of such self-processing peptides has allowed the production of stoichiometric levels of multiple gene products along with appropriate compartmentalisation. Szymczak and collegues (Ref. 92) made use of multiple 2A sequences, derived from different viruses, to derive a retroviral vector coexpressing the four members of the CD3 complex. When introduced into bone marrow of CD3-null mice, this led to efficient and stochiometric production of these four membrane proteins and correction of the multiple gene deficiency, resulting in restoration of T-cell function.

In vivo selection

The recent success in treating patients with X-linked SCID (SCID-X1) has given new heart to the gene therapy community (Refs 93, 94, 95; reviewed in this journal in Ref. 96). Much of the success has depended on improvements in transduction procedures and vectors, as discussed above. However, it is likely that at least two other factors have also contributed. First, in the SCID-X1 studies, there is a lack of immune response to the transgene, since the patients are immunodeficient. Second, and of importance to this review, expression of the therapeutic transgene (the cytokine receptor common γ-chain) leads to a profound survival and proliferative advantage in gene-corrected cells. For several disorders where HSC gene therapy might be curative (e.g. thalassaemia, chronic granulomatous disease),

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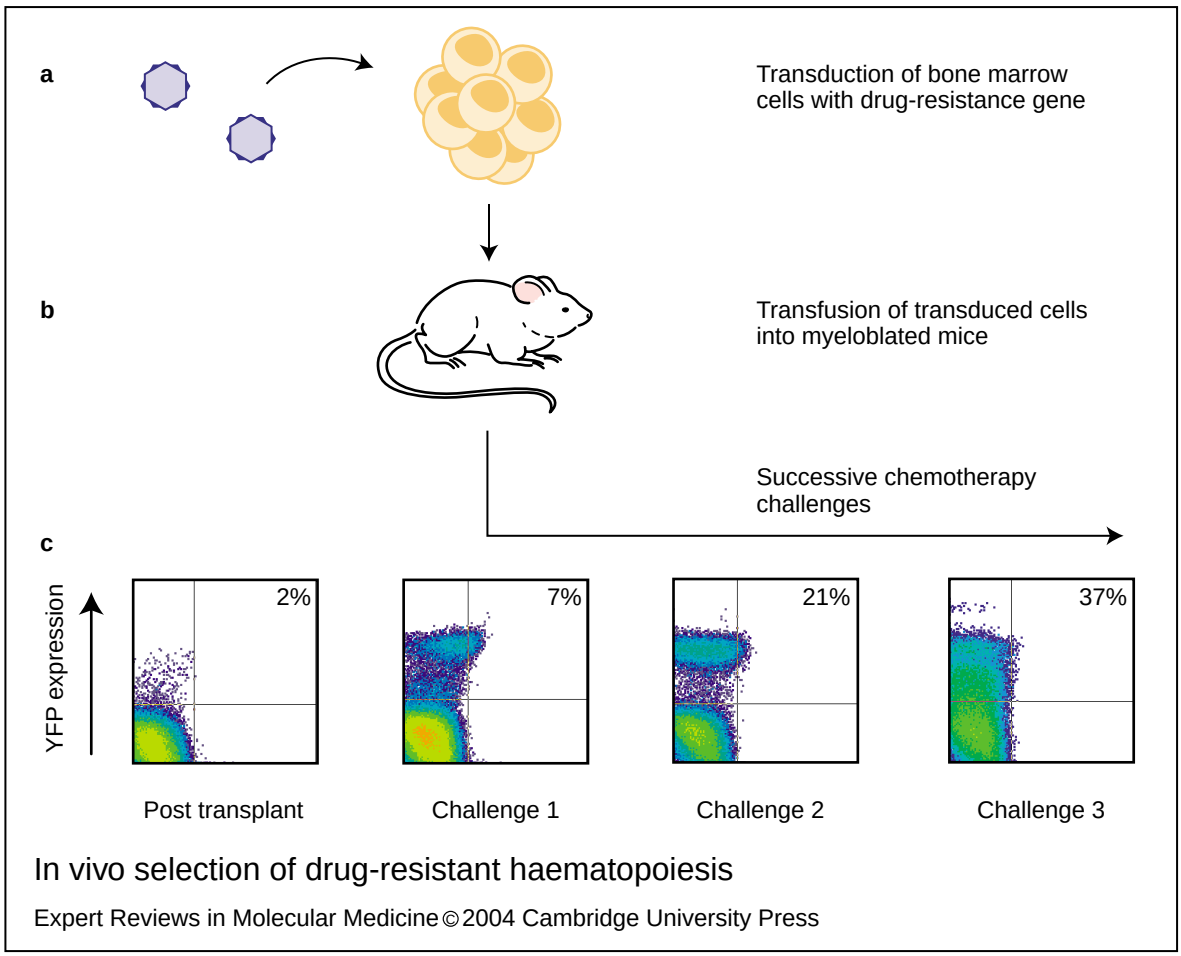

Figure 3. In vivo selection of drug-resistant haematopoiesis. In many applications of gene therapy to haematopoietic stem cells, it might be important to confer a selective advantage on transduced cells, and this might be achieved by the endowment of drug resistance. (a) Bone marrow cells harvested from mice pre-treated with 5-fluorouracil (to enrich for primitive cells) and pre-incubated in a cytokine cocktail (to induce cell cycling and facilitate retroviral transduction) are co-cultivated for two days with packaging cells for retroviruses carrying a drug-resistance gene (e.g. *MDR-1*) and a reporter gene (e.g. *YFP*). (b) Haematopoietic cells are then harvested from the coculture and transplanted into syngeneic recipients that have been myeloablated by total body irradiation. Following recovery of haematopoiesis in the transfused mice, successive rounds of low-dose chemotherapy (e.g. etoposide) are delivered. (c) Analysis of peripheral blood for YFP reveals how successive selective challenges increase the proportion of transduced cells in peripheral blood. Abbreviations: MDR-1, multiple drug resistance 1; YFP, yellow fluorescent protein.

there is unlikely to be an advantage to gene-corrected cells. It might therefore be important to confer an artificial advantage, and the endowment of drug resistance is one way in which this could be achieved (Fig. 3).

The selective survival in vivo of cells transduced by retroviral gene therapy has been shown in chemoprotection studies in which multiple rounds of drug administration were given (Refs 75, 77, 97, 98). It was observed in these studies that the level of chemoprotection increased with subsequent rounds of treatment and that the proportion of gene-modified cells increased concomitantly. Subsequent in vivo studies showed that bicistronic vectors coexpressing a therapeutic transgene in cohort with a drug-resistance marker facilitated selection of gene-modified cells expressing high levels of the therapeutic gene product (Refs 41, 99).

An important consideration in in vivo selection is the length of time for which selection can be maintained. Most of the drugs used for in

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vivo selection are not only cytotoxic but also mutagenic, and prolonged exposure of patients to such agents is likely to lead to the inadvertent induction of tumours (often leukaemias or lymphomas) by the procedure itself (i.e. iatrogenic tumours) (Ref. 100). Therefore, selection should ideally be achievable with the minimum number of exposures of patient to the selective agent. This requires that selection occurs at the level of the HSC, and presents problems with some agents. For example, methotrexate exhibits toxicity mainly to the committed progenitor compartments (Ref. 101), and selection with methotrexate tends to be transient, as non-gene-modified stem cells continue to contribute to haematopoiesis. This is at least in part due to the expression in more-primitive cells of nucleoside transporters that allow circumvention of de novo nucleotide synthesis and thus resistance to methotrexate toxicity. Inclusion of a nucleoside transport inhibitor in selection protocols leads to enhanced myelotoxicity with methotrexate and to improved selection in a mouse model. This selection included primitive cells, as shown by improved levels of gene-modified cells in secondary transplanted animals (Refs 102, 103). However, selection remained transient, with a gradual fall in transduced cells post-selection. A similar finding was made in a primate study where only transient selection was achieved in rhesus macqaques, with levels of gene-modified cells returning to baseline within 3 weeks post-selection (Ref. 104).

Similar problems could limit the utility of MDR-1 for long-term selection. Primitive haematopoietic cells, including stem cells, express ABC transport proteins (Refs 105, 106). Thus, very primitive cells are more resistant to MDR-1 substrate drugs than their differentiated progeny. This is reflected in the data obtained in in vivo experiments with *MDR-1* gene transfer. It is clear from preclinical experiments in mice and dogs that *MDR-1*-transduced haematopoietic cells have an in vivo survival and selective advantage following exposure to appropriate chemotherapeutic agents (Refs 16, 19, 41). In clinical trials there are suggestions that MDR-1 might lead to selection in humans (Ref. 32) However, in animal studies, the selective effect has been transient, reflecting selection at a more committed stage of differentiation than the stem cell (Refs 41, 107). One solution could be to use a more aggressive regimen, with a view to overcoming endogenous resistance in stem cells, yet allowing survival of cells overexpressing exogenous MDR-1. However, some care will be needed if such a strategy is to be used. The selective drugs that can be used in combination with MDR-1 are nonspecific and can damage a wide range of cells and tissues. Indeed, in a study in dogs (Ref. 41), administration of taxol at moderately high levels resulted in the death of two out of three experimental animals. The third, treated at a lower dose of taxol, showed evidence of gene-modified cells post-selection despite these cells being undetectable before selection. However, at later time points, MDR-1 expression decreased and transgene positivity (by polymerase chain reaction) in peripheral blood declined markedly, suggesting selection at best in a long-lived progenitor compartment.

By contrast, mutant MGMT could offer a better solution. It is known that primitive haematopoietic cells express very low levels of MGMT and that these cells are highly sensitive to O^6-alkylating agents (Ref. 63). Several murine studies have shown convincing evidence for in vivo selection of cells expressing mutant MGMT following treatment of transplanted animals with O^6-beG in combination with either temozolomide or BCNU (Refs 75, 77, 97, 98, 99, 108, 109). Follow-up post-selection has been relatively short in most studies, so it is difficult to determine whether selection would have resulted in long-term expression of the transgene. However, high levels (60–80%) of transgene positivity can be achieved in secondary recipients of bone marrow from primary transplant hosts (Refs 77, 98, 108). This suggests that primitive cells were selected in those primary animals. Moreover, in a canine study, a high level of gene-modified cells has been achieved post-selection, and this has been maintained for up to one year (Ref. 110). Thus, mutant MGMT could provide a means of achieving long-term and stable selection of gene-modified cells in vivo.

Engineering drug sensitivity

As well as using gene therapy to increase the resistance of haematopoietic cells to cytotoxic drugs, it is also possible to engineer drug sensitivity. This strategy is often referred to as gene-directed enzyme prodrug therapy (GDEPT) or 'suicide gene therapy' (Ref. 111). Such strategies provide a means to eliminate a gene-modified graft in the event of an adverse effect such as graft-

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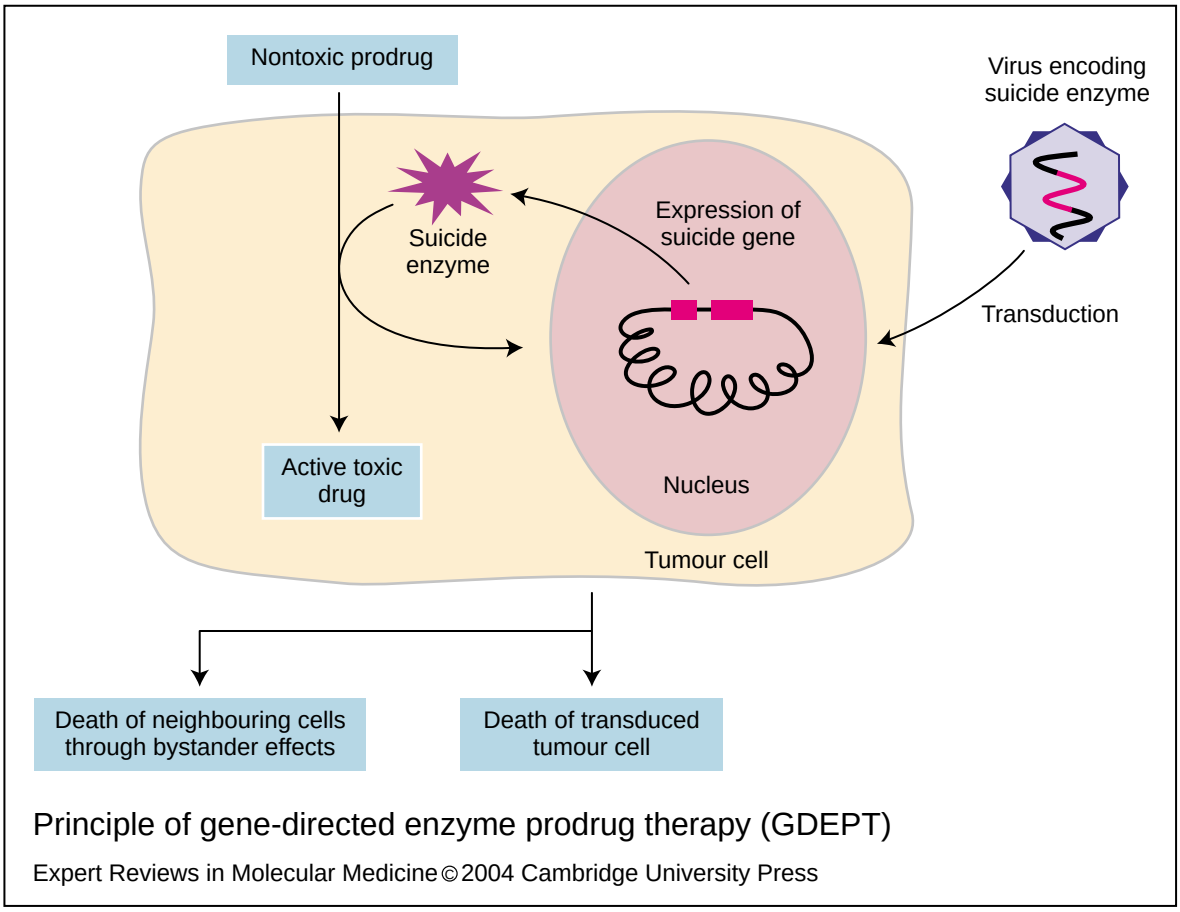
versus-host disease (GVHD), or provide a 'safety' mechanism to allow elimination of gene-modified cells in the event of an iatrogenic neoplasma such as a leukaemogenic event. A further application of GDEPT might be to use haematopoietic cells as a means of targeting bioactive proteins into tumours.

GDEPT: the concept

The underlying concept of GDEPT is that an otherwise nontoxic prodrug (or a drug with limited toxicity) becomes activated by an enzyme encoded by a transgene delivered by gene therapy, with a resulting cytotoxic effect. This strategy was originally developed as a means of specifically activating cytotoxic compounds within tumour cells. In this context, genetic transfer of an activating enzyme to tumour cells prior to delivery of the prodrug might be used to target cytotoxic effects to a tumour mass, sparing normal tissue (Fig. 4). Several enzyme–prodrug GDEPT systems are under study; these are listed in Table 3 and are discussed in greater detail in Ref. 112.

In the main, the activating enzyme in GDEPT systems is encoded by a cDNA or gene that is nonmammalian in origin – generally bacterial or viral. The obvious reason for this is that there is less likelihood of normal human cells expressing a comparative activity and thus converting the prodrug to a cytotoxic derivative. However, such an approach has to take into account the



Principle of gene-directed enzyme prodrug therapy (GDEPT)

Figure 4. Principle of gene-directed enzyme prodrug therapy (GDEPT). An appropriate vector (typically viral) is used to achieve insertion and expression of a prodrug-activating enzyme (see Table 3) and expression within tumour cells. Following administration of the otherwise nontoxic (or minimally toxic) prodrug, this is converted to an active, toxic metabolite in transduced cells. These are subsequently killed. Bystander, untransduced cells might also be killed following prodrug activation, by mechanisms that include direct transfer of activated drug through gap junctions, ingestion of apoptotic bodies from killed cells, effects on tumour vasculature, or immunological responses.

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Table 3. Gene-directed enzyme prodrug therapy (GDEPT) systems in current use

Enzyme	Prodrug (example)	Active metabolite	Ref.
Carboxylesterase	Irinotecan (CPT-11)	7-Ethyl-10-hydroxy-camptothecin	168
Carboxypeptidase G2	4-[(2-Chloroethyl)(2-mesyloxyethyl) amino]benzoyl-L-glutamic acid (CMDA)	4-[(2-Chloroethyl)(2-mesyloxyethyl) amino]benzoic acid (CMBA)	169
Cytochrome P450	Cyclophosphamide	4-Hydroxycyclophosphamide, which degrades into acrolein and phosphoramide mustard	170
Cytosine deaminase	5-Fluorocytosine	5-Fluorouracil	171
Herpes simplex virus thymidine kinase	Ganciclovir	Ganciclovir triphosphate	116
Nitroreductase 1	5-Aziridinyl-2,4-dinitrobenzamide (CB1954)	5-(Aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide	172

potential for an immune response to the exogenous protein, and indeed such responses to herpes simplex virus thymidine kinase (HSV-TK) have been reported in a clinical trial (Ref. 113). In GDEPT strategies aimed at transducing and sensitising tumour cells, immune reactions might potentiate the therapeutic response (Ref. 114). However, immunological complications might also lead to compromised therapeutic effectiveness (Ref. 113). One solution to this might be to express a protein of human origin. An example would be carboxylesterase, which can convert the chemotherapeutic drug irinotecan to its active agent SN38 (Ref. 115). Such an approach, however, will rely heavily on achieving a suitably large differential between levels of expression in normal tissue and those in gene-modified cells in order to minimise collateral toxicity.

In the haematopoietic system, the GDEPT strategy that has been most extensively tested has been the use of HSV-TK in combination with ganciclovir (GCV) (Ref. 112). GCV is widely used as an agent for the treatment of cytomegalovirus and herpes simplex infections. It is a poor substrate for mammalian nucleoside kinases, but it is efficiently phosphorylated to the monophosphate form by HSV-TK (Ref. 116). Subsequently, cellular kinases mediate its further metabolism to a toxic triphosphate derivative that is incorporated into the host-cell DNA, leading to killing of actively dividing cells.

GDEPT targeted at alloreactive T cells

The cell-cycle dependence of the killing action of HSV-TK–GCV led to the hypothesis that this system might facilitate deletion of alloreactive T cells in an allogeneic transplant setting. GVHD is a serious and potentially lethal consequence of allogeneic transplantation (Ref. 117). Strategies such as T-cell depletion of grafts or intensive immunosuppressive chemotherapy post-transplant can prevent or ameliorate GVHD (Refs 117, 118). However, this can lead to poor engraftment of donor haematopoiesis, and to prolonged immune deficiency and thus susceptibility of patients to infection [either exogenous or endogenous, such as reactivation of Epstein–Barr virus (EBV) and subsequent development of EBV-induced lymphoma] (Ref. 118). Moreover, where a patient is being transplanted in order to treat neoplastic haematopoietic disease (i.e. leukaemia), lack of an alloreactive graft-versus-leukaemia (GVL) effect leads to higher incidences of disease relapse (Ref. 119). For these reasons, several studies have examined whether a GDEPT approach might allow infusion of donor T cells in a situation that confers the capacity to delete such cells in the event of GVHD.

Early studies provided promising evidence of the utility of HSV-TK to rescue mice from GVHD post-transplantation (Refs 120, 121, 122). On the basis of these studies, clinical trials were undertaken in patients receiving allogeneic transplantation as an integral part of treatment

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for leukaemia. In the first report of such a study, eight patients were infused with donor lymphocytes in an attempt to control either disease relapse or EBV-induced lymphoma (Ref. 123). These lymphocytes were transduced with a retrovirus expressing HSV-TK. In five of the patients, a GVL effect was seen, with either complete or partial responses to treatment. Of these five patients, three also developed GVHD and were treated with GCV, resulting in complete deletion of gene-modified T cells and remission from disease in two patients, and a partial response in the third. In a related trial, patients received HSV-TK-modified T cells concurrently with a T-cell-depleted allogeneic bone marrow (Ref. 124). Three patients developed acute GVHD, of which two were successfully treated with GCV and the third required a combination of GCV and further immunosuppressive treatment; a fourth developed chronic GVHD that was also resolved with GCV.

Although these trials provided proof-of-principle of the potential of GDEPT to allow manipulation of GVL and GVHD in transplant patients, it became clear that several factors needed to be optimised. One of these stems from the observation that resistance to GCV is associated with cryptic splicing and subsequent deletion of sequences from wild-type HSV-TK (Ref. 125). Cells harbouring such a deletion exhibited a selective advantage in patients treated with GCV. Introduction of conservative point mutations into HSV-TK eliminates the splicing and could overcome this limitation of the wild-type gene (Ref. 126). Moreover, the introduction of multiple amino acid changes has resulted in the development of an enhanced version of the enzyme that confers elevated sensitivity to GCV (Refs 127, 128).

Akin to what has been previously described in studies of HSC gene therapy, it seems that the gene-transfer process might compromise T-cell function (Refs 129, 130, 131). Changes to in vitro culture conditions, including alternative cytokines, shorter-term culture and perhaps the use of lentiviral vectors [which require reduced culture times and less cytokine stimulation of target cells in order to efficiently transduce T cells (Ref. 132)] are being investigated as a means to achieve optimal transduction with minimal disruption to the target cells (Refs 130, 133). Similarly, the scheduling of GCV administration to patients merits close attention: early administration post-transplant might selectively delete alloreactive cells, leading to prophylactic treatment of GVHD (Refs 134, 135). Balanced against this is the need to maintain the alloreactive GVL effect, and murine studies suggest that a compromise in scheduling of GCV might achieve this (Ref. 136).

GDEPT targeted at macrophages

Macrophages constitute another potential target for GDEPT. The direct role of macrophages in tumourigenesis remains controversial as they display both a growth-promoting phenotype and tumouricidal activity (Ref. 137). Furthermore, despite forming a significant proportion of the solid tumour mass, it is unclear whether activated macrophages per se are potent enough to mediate a significant therapeutic effect. One approach to augment the antitumour potential of macrophages might be to endow them with a GDEPT capability with a view to harnessing a bystander effect, whereby cells surrounding gene-modified macrophages are exposed to a cytotoxic agent following macrophage-dependent activation of a prodrug.

One major caveat to this approach surrounds the behaviour of systemically infused macrophages in animal models and patients. Although such cells might locate to tumour sites, the efficiency with which they do so can vary greatly, with a majority of macrophages locating to other sites, notably the lungs, liver and spleen (Ref. 137). Uncontrolled expression of a prodrug activator in such tissues would thus lead to a risk of extensive collateral damage on administration of the prodrug. The local administration of macrophages within tumours could overcome this limitation, but such an approach would not be applicable to disseminated disease. An alternative approach has used bispecific antibodies that bind both macrophage and a 'tumour-specific' antigen (Refs 138, 139). This has been shown in murine models to enhance tumouricidal activity and might have a role in enhancing homing to disease sites.

Alternatively, it might be feasible to restrict expression of prodrug activators to macrophages that are located within the tumour environment. The best characterised of these approaches seeks to exploit the observation that macrophages tend to home to areas of hypoxia in tumours, and express several hypoxia-related genes, including some involved in glucose metabolism

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and angiogenesis (Ref. 140). In these regions, the activity of the transcription factor hypoxia-inducible factor (HIF) is upregulated in macrophages. HIF in turn binds to hypoxia-responsive elements, leading to upregulation of transcription of hypoxia-responsive genes. Thus, expression of a GDEPT enzyme by a hypoxia-responsive promoter might restrict prodrug activation to within a tumour mass and thus avoid systemic toxicity. One early study has shown hypoxia-dependent expression of human cytochrome P4502B6 in an in vitro tumour spheroid model (Ref. 140), suggesting this approach might have utility. However, this has not yet been tested in an in vivo model, and concerns remain over whether basal levels of expression in normal tissues might be high enough to lead to significant levels of activated drug outside the tumour mass and to subsequent toxicity. Moreover, other physiological stimuli, such as insulin or cytokines, might also upregulate HIF activity and thus lead to increased collateral toxicity (Refs 141, 142, 143, 144). One potential solution to this problem might be to use a bioreductive prodrug. Such drugs, when activated by an appropriate reductase, can cause DNA damage to cells under hypoxic conditions (Ref. 145). However, under normoxic conditions, they are rapidly re-oxidised to a nontoxic form. For example, hypoxia-dependent expression of the cytochrome *c* P450 reductase can be combined with activation of the bioreductive nitroimidazole RSU1069 to achieve efficient killing of target cells under hypoxic conditions in vitro (Ref. 146). However, as with any genetic approach, careful in vivo modelling will be essential to evaluating the potential of this strategy.

GDEPT targeted at bone marrow progenitors

Finally, one further means of targeting GDEPT to tumours via the haematopoietic system has emerged from a study where bone marrow progenitors were transduced with a lentiviral vector containing regulatory elements from the *Tie2/Tek* gene, which is preferentially expressed in endothelial cells (Ref. 147). In tumour-bearing mice, this vector marked a distinct set of haematopoietic cells that homed to the tumour and interacted with vascular endothelial cells. In combination with HSV-TK expression in these cells, GCV administration led to significant reductions in tumour growth and inhibition of tumour angiogenesis. This haematopoietic approach to targeting cytotoxic therapy to the tumour vasculature holds promise and merits further and careful analysis.

Safety

When proposing the genetic modification of HSCs, and particularly when considering enhancing resistance to antitumour agents, some thought should be given to the prospects for induction of iatrogenic tumours (Ref. 148). Retroviral vectors contain powerful transcriptional enhancers and these can influence the activity of promoters in the region of the insertion site. Recently, two patients treated by retroviral gene therapy for SCID-X1 developed a proliferative disorder that has been attributed, at least in part, to insertional activation of an oncogene by the retroviral vector (Refs 149, 150). In addition, in two mouse models, iatrogenic leukaemia has been described as a result of retroviral transfer of a therapeutic or marker gene to HSCs (Refs 42, 151). In the first of these, transfer of the gene encoding MDR-1 to murine bone marrow cells was associated with the development of neoplastic disease in recipient animals (Ref. 42). No previous experiments using *MDR-1* gene transfer had indicated any such problem and indeed subsequent experiments in a primate model, using the same vector as the murine study, revealed no evidence of myeloproliferative disease in those recipients (Ref. 152). Moreover, overexpression of MDR-1 in a murine cell line conferred drug resistance but did not affect other parameters such as growth factor response, differentiation or growth rates (Ref. 153). It seems likely that the oncogenic effects seen were related to the high multiplicity of infection (MOI) and the subsequent large number of retroviral insertions that resulted in gene-modified cells. In the other murine study, oncogenic insertion of what was presumed to be an innocuous marker gene – the truncated nerve growth factor receptor – was directly implicated in the development of a leukaemic clone (Ref. 151). In this study, the authors clearly showed that an oncogene (*evi-1*) was upregulated as a consequence of insertional activation by the retroviral vector. The previous observation that *evi-1* is not acutely leukaemogenic also led to the authors suggesting that an interaction between the transgene and the activated oncogene might be an important parameter in the neoplastic development. Furthermore, the observation that

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deletion of MDR-1 activity results in reduced levels of polyposis in $APC^{min-/-}$ mice [which carry a nonsense mutation in the adenomatous polyposis coli (*APC*) gene and have a consequent increased susceptibility to tumourigenesis in the colon and small intestine] suggests that an as-yet-occult activity of MDR-1 might exist that could contribute to transformation under some circumstances (Ref. 154). Clearly, for any putative therapeutic transgene to be used, careful preclinical assessment of potential adverse effects will be required.

The extent of vector integration into haematopoietic and other target cells is dependent on the MOI of the vector. Clearly, the more vector a target cell is exposed to the greater the risk of multiple integration events, and thus of any one cell receiving a proleukaemic or other potentially pathogenic integration. An emphasis on achieving high levels of transduction has contributed to the phenomenon of multiple integrations, since high levels of transduction occur with high MOIs (Ref. 148). Indeed, a recent study using a retroviral vector demonstrated the relationship between transduction frequency and proviral copy number (Ref. 155). A transduction frequency of 30% or less led to one proviral copy per transduced cell; higher transduction frequencies resulted in greater numbers of integrations, with some clones assessed having more than ten proviral copies. Such high numbers of proviral copies per cell should be considered undesirable and it seems likely that moderate, rather than high, transduction frequencies might be preferable. Under such circumstances, the in vivo selection of gene-modified cells might prove important in order to attain a therapeutic response.

Research in progress and outstanding research questions

The principle of chemoprotection/chemoselection needs to be rigorously tested in clinical trials. Previous trials using MDR-1 suffered from poor transduction conditions and suboptimal vectors. With the improvements in gene-transfer technologies over recent years, the time is right to re-examine this area. The best candidate for clinical investigation of chemoprotection is MGMT, and several trials are planned. These will be initially undertaken in patients undergoing chemotherapy for the treatment of solid malignancies – primarily glioma and melanoma. Important parameters to be measured will include levels of transduction and engraftment of gene-modified cells, as well as the effects of subsequent chemotherapy on overall haematopoiesis and, in particular, on transduced cell numbers (i.e. is there evidence for in vivo selection?). If evidence of substantial protection of haematopoiesis is obtained, then it might be appropriate to extend these studies to examine the potential for chemotherapeutic dose intensification or escalation. In vivo selection with MGMT or any drug-resistance gene is unlikely to be tested clinically outside of the context of cancer for some time. Prior to this, clear indications will be required that the gene-transfer process and the drugs used for in vivo selection show an acceptable safety profile (see below).

Many of the shortfalls in the maintenance of the T-cell repertoire that characterised early trials of HSV-TK for the control of GVHD have been successfully addressed in mouse studies, as have important issues surrounding scheduling of GCV administration. It would seem appropriate to begin further trials in this area; again, these are planned. Important parameters to be assessed will include the maintenance of the T-cell repertoire in transplanted patients and, importantly, of the graft-versus-infection and GVL capacity of the gene-modified T cells.

The use of haematopoietic cells as vectors for the delivery of prodrug-activating enzymes to tumours is in a much earlier stage of development. The major obstacle to this approach is the design of vectors and strategies that will lead to tumour-specific activation of the prodrugs. The use of hypoxia as a means of controlling transgene expression might facilitate this, but it must be extensively tested in in vivo models, with considerable emphasis on the specificity of transgene expression and prodrug activation. Some studies using bispecific antibodies suggest that it might be possible to target cells more effectively into tumour masses. However, these data are preliminary and careful analysis of cell trafficking and transgene expression will be required in vivo.

Safety is of paramount concern in all gene therapy applications. Where a therapeutic application involves the induction of resistance to chemotherapeutic agents, this concern is understandably amplified. For this and other reasons, clinical trials of drug-resistance gene therapy will be conducted in patients already being treated for malignant disease. However,

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there is much room for improvement of the safety of current vectors and transduction protocols (Refs 156, 157). The reported adverse effects in patients have occurred following retroviral gene transfer, and it has been documented that such vectors preferentially integrate near transcriptionally active genes (Refs 158, 159). However, lentiviral and adeno-associated virus vectors also preferentially integrate in transcriptionally active regions (Refs 160, 161), and there is no guarantee that these will prove to be any less prone to causing insertional activation of host genes. The development of vectors carrying isolator regions, which could facilitate efficient expression of a transgene while minimising effects on the surrounding genome, is an attractive proposition; so also is the possibility of screening stem cells for potentially dangerous insertions prior to transplantation. However, neither of these is currently technically feasible, and each approach will require a considerable effort in order to come to fruition. Meanwhile, the risk of adverse effects in patients could be minimised by careful modelling in murine systems, leading to a better understanding of the interactions between transgenes and insertion sites. This should be coupled with transduction protocols that lead to integration of a single copy of vector per repopulating cell and with administration of a minimal number of transduced stem cells compatible with long-term effectiveness of treatment. These measures will not eliminate the risk of insertional mutagenesis but should substantially reduce it. Finally, in determining whether it is appropriate to test any given genetic therapy in patients, serious thought should be given to the risk–benefit analysis. For some patients, the consequences of not treating their condition by genetic means might be worse than the risk associated with gene therapy.

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