The Academy of Medical Sciences | FORUM

Safer Medicines Report

Gene Therapy working group report

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Executive summary and recommendations

Gene therapy: expression vectors delivered by naked nucleic acids or vector particles

Gene therapy is a novel way of treatment for a variety of diseases by transferring specific, functional genetic material into somatic target cells. Delivery and expression of therapeutic, preventive or diagnostic genes is achieved by the use of 'vectors'. These are complex biological products – naked nucleic acids, non-viral or viral vector particles – that are designed to transfer and express the incorporated gene(s) (commonly termed transgenes) for an intended medicinal purpose. Generally, viral vectors used in gene therapy are replication-incompetent viruses expressing single transgenes. However, in certain cases, recombinant and conditionally replication-competent viruses are used, e.g., to kill tumor cells *in vivo*, with the viral genome acting as the therapeutic 'gene'.

Several replication-incompetent expression vectors are in current clinical use. These include naked plasmid DNA, non-viral vectors (plasmid DNA delivered in a mixture with transfection reagent), gammaretroviral vectors, adenoviral vectors, adeno-associated viral (AAV) vectors, pox-viral vectors and herpes viral vectors. In the main such gene therapy medicinal products are targeted to (i) monogeneic inherited diseases, (ii) cancer, (iii) cardio-vascular diseases, and (iv) infectious disease, mainly HIV infection.

Beneficial gene therapy approaches and related theoretical or practical safety risks

It is widely anticipated that gene therapy will provide beneficial treatment in monogeneic diseases such as severe combined immunodeficiency (SCID), will lead to improvement in survival rates and quality of life of certain cancer patients, and will have beneficial effects in cardiovascular diseases. Phase II/III or phase III clinical trials will show whether adequate clinical endpoints can be attained. First licensing applications are expected within the next few years.

A number of adverse reactions are now being recognized as causally linked to particular gene therapy approaches. They include: the toxicity of adenoviral vectors if used systemically in doses above 10^{13} particles; the induction of leukemia following infusion of murine leukaemia virus (MuLV) vector-modified blood stem cells in children less than 6 months of age, when a strong selective advantage is conferred upon the modified cells; the potential development of auto-immunity in a preclinical setting following administration of high titer adeno-associated virus (AAV) vector expressing an endogenous erythropoietin gene. Such observations will need to be assessed in detail so that appropriate actions are undertaken to protect patients enrolled in clinical trials from such adverse 'side effects'. In general, however, gene therapy has been shown to be relatively safe: an analysis of the FDA database on serious adverse events reported from gene therapy clinical trials found no clear association of certain adverse events with particular vectors or clinical gene transfer approaches.

General and particular safety concerns to be investigated with a view to better safety of gene therapy

The principal safety concerns in gene therapy are:

(i) contamination of replication-incompetent viral vectors with replication competent virus derived from vectors, or the combination of vectors, within the so-called 'packaging cells' used for their production;

(ii) insertional mutagenesis predisposing to malignancy caused by certain integrating vectors;

(iii) adverse systemic effects induced by therapeutic proteins released from the genetically modified, vector-harbouring cells *in vivo*;

(iv) biodistribution of vector to non-target cells or tissues, particularly germline cells, the modification of which is legally prohibited in the European Union (EU).

However, since gene therapy approaches are diverse, there is a diversity of the theoretical safety risks, leading potentially to adverse effects, to be considered. A general outline of the safety issues in gene therapy is probably therefore pointless. For the

sake of rationalization, it is appropriate to consider collectively the approaches in:

(i) uses of conditionally-replication competent, oncolytic viruses,

(ii) systemic and local use of replication-incompetent viral vectors,

(iii) use of particular genetically modified cells, particularly haematopoietic stem cells,

(iv) preventive uses of naked plasmid DNA, non-viral vectors or vectors as vaccines, where the main avenues for improving the safety of gene therapy are identifiable and open to further investigation. They are respectively: (i)

- the targeted replication of the oncolytic viruses;
- the 'non-target cell' effects of vector;
- the phenotypic adverse changes that could be introduced by genetic modification;
- the potential induction of auto-immune phenomena by the gene transfer vaccine.

As gene therapy development is in its infancy and clinical (gene transfer) approaches are experimental and numerous, a comprehensive or detailed description of all safety risks is impractical. [Examples of particular safety risks are given in the chapters following this summary]. In general, it is necessary, however, to develop specific control and test methods in conjunction with development of particular vectors, coordinated with respect to the clinical approach taken and disease status of patients treated. For example, the induction of leukemia during SCID-X1 treatment in children under 6 months of age has led to the development of several control and test methods previously not at hand.

Scientists are now able to mimic leukemia development in a mouse bone marrow transplantation model. Here, bone marrow stem cells are genetically modified with murine leukaemia virus-derived vectors, similar to those used in the clinical gene therapy of SCID-X1. Cancer develops within months due to chromosomal integration of a high copy number of murine leukaemia virus-derived expression vectors within a particular bone marrow stem cell. The integration of several expression vectors is necessary for insertional mutagenesis of particular oncogenes to elicit leukemia in this model.

Regulatory support for gene therapy

Regarding regulatory support, the number of groups concerned with gene therapy is sufficient and allows practical support. The groups concerned with developing regulatory guidance for the facilitation of gene therapy include: WHO Clinical Gene Therapy Monitoring Group, ICH Gene Therapy Discussion Group (ICH-GTDG), Ad hoc CHMP Gene Therapy Expert Group (GTEG) and now the CHMP Gene Therapy Working Party (GTWP) at the European Medicines Evaluation Agency (EMEA), Gene Therapy Societies and various Ad Hoc Committees for standardisation of gene therapy products and the development of reference materials. These, together with central ethics committees and competent authorities in European member states, including the (EMEA), provide good regulatory advice.

One third of all gene therapy clinical trials carried out so far have taken place in Europe – mainly in the UK, Germany, Sweden, France, The Netherlands, Italy and Spain – with most of the other two thirds having taken place in the USA. To-date, however, no gene therapy medicinal products have been licensed in Europe. Nevertheless, the EMEA orphan drug status for products used for treating rare diseases supports the use of some gene therapy medicinal products without full clinical trial evaluation.

Currently, safety issues are being discussed as within a 'moving target' of scientific evidence and advice as many new gene therapy medicinal products are being evaluated, both in pre-clinical and clinical trials, and clear and stable patterns of efficacy and safety frequently remain to be established. Taking these uncertainties into account, The Working Group took as the basis of their Report specific illustrative examples of clinical gene transfer and considered how the safety of gene therapy might be improved.

Proposals and Recommendations

General recommendations

- Assessment of the safety of gene therapy medicinal products must be based on scientific findings and critical unbiased judgement of experimental data/evidence, and should not be influenced by

political expediency, legal advice or regulatory sensitivities/prejudices.

- Safety testing must be specific for individual vector types and all tests must be well-validated using suitable reference materials.
- All tests that are carried out must be relevant to the actual and perceived safety risks of vectors. Unnecessary tests for non-existent risks should be avoided.
- Most safety testing that is currently applied to gene therapy medicinal products is carried out by manufacturers and academic or pharmaceutical developers. Independent testing laboratories capable of performing platform safety studies addressing common important risks are few and far between. More funding should therefore be allocated to support independent testing of gene therapy medicinal products to ensure safe use of gene therapy medicines and to support future developments.

Vector design and manufacture

- For replication deficient viral vectors, manufacturing strategies to prevent the generation of replication competent viruses (RCR) are paramount. Every effort should therefore be made to ensure the design of vector constructs and viral helper genes/ constructs will remove completely the possibility of forming RCR.
- In the case of integrating viral vectors, design features to prevent or minimize the possibility of insertional oncogenesis in target cells are increasingly important. Research in vector modifications, e.g., the use of insulator sequences, self-inactivating (SIN) mutations, is to be encouraged and supported.
- For *in vivo* applications, vector targeting to target cells is an important goal. Research into ways of modifying vectors so that they interact only with intended target cells/tissues should continue and is strongly supported.
- For replication-competent vectors, specific safety requirements need to be developed to ensure safe use in the absence of transmission to third parties.

Pre-clinical/non-clinical safety testing

- General toxicity tests are frequently of little value for gene therapy medicinal products; appropriate animal models are often lacking. However, non-clinical testing in validated animal models for specific properties related to safety/efficacy is essential and should be strongly supported. Biodistribution studies are essential for vectors for use in *in vivo* applications.
- Validated physical and biological methods to quantify viral vector particles (or impurities such as replication competent viruses), are essential for product characterization and consistency and for defining appropriate dosages and/or transduction efficiency. The application of modern technologies such as NAT in this respect is to be encouraged.
- In the absence of *in vivo* endpoints, test methods to measure surrogate markers related to vector activity should be developed.
- When serious adverse effects are seen in participants of gene therapy trials, the development of appropriate (novel) test procedures that mimic such effects at the pre-clinical level of safety testing should be encouraged.

Clinical monitoring

- Long-term follow up of patients in clinical gene transfer trials is necessary to account for slowly developing adverse effects of treatment, e.g., leukaemia. More information could be obtained from patients undergoing gene therapy by conducting more intensive studies of responses, for instance employing genomics or proteomics for devising more sensitive testing of adverse effects.
- Studies of the interactions of vectors and transgenes with the immune system are currently too few and underfunded. More immunological research in patients receiving gene therapy medicinal products is urgently needed.

Chapter One - *Gene transfer/therapy: definition and approaches*

Definitions

1.1 Gene therapy medicinal products (GT-MPs) may consist of viral or non-viral vectors or nucleic acid used for genetically modifying human somatic cells *in vivo* (*in vivo* strategy) or may consist of *ex vivo* genetically modified autologous, allogeneic or xenogeneic cells (*ex vivo* strategy). Viral vectors (replication incompetent viruses able to transfer a therapeutic gene), non-viral vectors (DNA encompassing the therapeutic gene mixed with transfection reagents), 'naked' DNA or conditionally-replicating microbes (recombinant adenovirus, recombinant Salmonella) are currently used for genetically modifying a cell *in vivo* or *ex vivo*. Gene therapy medicinal products may also involve replication competent, recombinant microbes used for purposes other than prevention or therapy of the infectious disease they cause (*in vivo* strategy).

1.3 This scope of GT-MPs is in agreement with the variety of GT-MPs covered by the current European Note for guidance on the quality, preclinical and clinical aspects of gene transfer medicinal products (CPMP/BWP/3088/99). The application of antisense nucleic acid itself, which does not encompass a gene and which is used without the intention of genetically modifying cells, e.g. anti-sense RNA for the treatment of Cytomegalovirus-induced retinitis, is not considered to belong with GT-MPs, but rather to molecular medicine.

Characteristics of GT-MPs

1.4 The nucleic acid construct (encompassing promoter, enhancer and other regulatory sequences, one or more cDNAs including a poly-adenylation signal and a coding region or a region specifying an RNA product) has been variously termed 'expression construct', 'expression vector' or 'transfer vector'. Plasmid DNA used as naked DNA or as part of a non-viral vector is produced from *E. coli* bacteria. Viral vectors are generally produced by mammalian 'packaging' cells in culture. The transgene product is normally a protein with a specific function, or is an antigen. It may also be a specific RNA, e.g., ribozyme. The 'delivery vehicle' which allows transfer of the expression vector (and thereby the transgene(s)) into cells determines the structure of the expression vector and can be of viral or non-viral origin. Viral vector particles, non-viral vectors and free nucleic acids (e.g., naked DNA) which are currently in clinical use are summarized in Table 1. Further details of vectors are given in Section 3.

Clinical gene transfer

1.5 Gene therapy medicinal products are intended for the treatment, diagnosis or prevention of a variety of diseases. Gene therapy and somatic cell therapy using genetically modified cells are best considered under the term 'clinical gene transfer'. During clinical gene transfer, nucleic acids containing one or more transgenes are transferred into cells in order to restore normal cellular functions or to add new ones. For example, inherited monogeneic disorders such as haemophilia and cystic fibrosis are due to somatic mutations in the genes encoding Factor VIII/IX and CFTR proteins, respectively, and which result in their malfunction.

1.6 Vector-mediated transfer of the appropriate un-mutated gene, together with appropriate regulatory sequences for control of protein expression, to relevant somatic cells should provide some measure of the biological or physiological function lacking in monogenic disorders. Gene therapy can also be applied to cancer treatment. For example, vector-mediated transfer of a gene encoding an enzyme that catalyses conversion of a chemical (pro-drug) into a toxic metabolite into tumour cells should provide the means for their destruction. As an alternative approach, introduction of a gene(s) encoding a biologically-active protein(s), e.g., cytokine(s), should provide activating signals that stimulate

the immune and/or other physiological systems to eliminate tumour cells.

1.7 Examples of *in vivo* strategies include intra-muscular injections of naked (plasmid) DNA, intra-tumoral inoculation of non-viral vectors, infusion of adenoviral vectors via the intra-hepatic artery or intra-muscular injection of adeno-associated virus (AAV) vectors. The transgene is thus delivered to human somatic cells *in vivo* where it is expressed *in situ*. An example of a live recombinant micro-organism used in clinical gene transfer is a replication competent adenovirus not carrying any transgenes. They are inoculated, e.g., into local head-and-neck tumors and, in conjunction with conventional chemotherapy, may effect local tumor regressions.

1.8 Clinical gene transfer may follow the *in vivo* strategy, as exampled above, or an *ex vivo* one. During *ex vivo* gene transfer, somatic cells are genetically modified in cell culture by viral or non-viral vectors, as appropriate. The modified cells are grown in culture and then injected into patients. Genetically modified autologous cells, such as CD34-positive cell that contain hematopoietic stem cells or progenitor cells, may, following engraftment, yield large numbers of long-lived modified daughter cells that provide beneficial effects.

1.9 Irradiated allogeneic cells may be used as tumor vaccines; these induce an immune response directed against tumour cells. The clinical use of xenogeneic primary cells is considered unsafe because of the theoretical risk of transmitting xenogeneic infectious agents to humans. However, intrathecal inoculation of xenogeneic (murine) retroviral packaging cells that produce retroviral vectors expressing a 'suicide' transgene, e.g., the *H*erpes *S*implex *V*irus-derived *t*hymidine *k*inase (*HSVtk*) gene (HSVtk converts co-administered pro-drugs (ganciclovir) into toxic metabolites) has been used in glioblastoma patients.

1.10 Although, clinical gene transfer has the potential to treat a variety of diseases, to-date there has been relatively few efficacious outcomes in gene therapy clinical applications. Numerous technical and physiological problems concerning vector targeting, anatomical barriers, immune responses, efficiency of gene transfer and appropriate regulation of transgene expression, have proved stubborn obstacles to overcome to attain success in the clinic. To overcome such obstacles and build towards safe and efficacious treatments for specific diseases, a new scientific area of vector technology has been established.

1.11 Since gene transfer is largely restricted by the cell membrane, 'vectorologists' have focused on biomaterials and viruses for constructing vectors that efficiently traverse this membrane. Naked DNA inoculated intra-muscularly or intradermally is spontaneously, but inefficiently, taken up by mammalian cells *in vivo*. Delivery can be improved by using instead of needle inoculation, a gene gun (gold particles coated with the plasmid DNA) or a jetgun (plasmid/helium gas under pressure).

1.12 Further improvements for delivery and transfer of non-viral vectors have relied on combinations of DNA with biomaterials, e.g., cationic lipids, which markedly increase transfer of DNA into cells. However, relatively speaking, gene transfer by this means is much poorer than that achieved with viruses, which have evolved to efficiently package and then transfer their genomes into host cells. Thus, viruses have provided the 'building blocks' for many vectors currently in use for gene transfer. However, viruses, while offering a broad 'palette' from which to create viral vectors suited for different medicinal purposes, are very variable in structure, tropism and pathogenicity. Targeting genes or their expression into specific cell types or tissues remains a principal aim for vector design. Viral vector pseudotyping using heterologous viral envelope proteins, such as the vesicular stomatitis virus G-protein (VSV-G), should, besides increasing efficacy, aid in this endeavour.

Chapter two - *Gene transfer vectors for clinical applications*

General considerations

2.1 For clinical use, vectors should exhibit a spectrum of desirable properties against the disease targeted (e.g. highly effective transfection or transduction, appropriate transgene expression level) without manifesting undesirable ones (e.g. high toxicity or immunogenicity, oncogenic activity)[1–3]. Desirable properties are inherent in several viruses, including retroviruses, adenoviruses, small DNA viruses, herpes viruses and poxviruses (Table 1). Since these viruses are often human pathogens, for safety reasons viral genes conferring replicative ability and virulence are removed to render derived vectors replication-deficient. Nevertheless, design and manufacture of replication-deficient viral vectors as GT-MPs is challenging since they mostly involve using mammalian cell lines transfected with complementing or 'helper' viral genes to enable vector propagation to take place; such usage could result in contamination with replication competent viruses (RCV) akin to pathogenic wild-type strains. It is therefore crucial that viral vectors are well-characterised and stringently tested at the pre-clinical level to assure freedom from RCV, other adventitious agents, e.g., mycoplasma, fungi, viruses, and mammalian cell contaminants such as chromosomal DNA and cellular proteins[1].

Vector types

Non-viral vectors and plasmid DNA

2.2 Genes may be readily incorporated into plasmid DNAs, which can in high yields be prepared by fermentation and lysis of known and safe *E.coli* strains. Purified DNA in its correct (supercoiled) form, free from endotoxins and residual bacterial DNA, RNA and protein contaminants, can be prepared from the lysate in compliance with GMP. Thus naked plasmid DNAs are considered less likely to raise as many safety concerns as viral vectors, but without targeting ligands to increase specificity, their transfection efficiency is relatively low , often requiring considerable amounts of pharmaceutical grade plasmid DNA for clinical gene transfer.

2.3 Plasmid DNA can be delivered by needle or catheter-directed injection, by gene gun, by

Table 1. Comparison of viral vector characteristics

Characteristic	**AAV**	**GV**	**LV**	**AdV**	***Vector*** **Gutless AdV**	**CRSd**	**HSV**	**Pox**	**SFV**
Capacity	4.5 kb	8 kb	18 kb	8–12 kb	32 kb	(8–12 kb)	20 kb	.25 kb	5 kb
Purity/stability	hi/hi	lo/lo	lo/lo	hi/hi	hi/hi	hi/hi	hi/hi	hi/hi	hi/hi
Transduction:									
Dividing cells	+	+	+	+	+	+	+	+	+
Non-dividing cells	+	−	+	+	+	+	+	+	+
Integration	+/−	+	+	+/−	+/−	+/−	−	−	−
Efficacy	hi	lo	lo	lo	hi	hi	hi	hi	hi
Transgene expression	m/l	m/l	m/l	s/m	s/m	(s/m)	s/m	s/m	s
Immunogenicity	+	−	−	+	−	+	+	+	−
Toxicity	−	−	−	+	+	+	+	+	+
In vivo/ex vivo use	1/1	2/1	1/1	1/2	1/2	1/2	1/2	1/2	1/2

KEY: hi = high; lo = low; s = short; m = medium; l = long

electroporation, and can be administered by food uptake. Plasmid DNA has been used in clinical trials for a variety of therapies; it is currently used in about 11% of the clinical trials worldwide. These trials include vaccines for prevention of HIV-1, HSV-1, malaria, influenza A virus, HBV, and HPV (see Section 5). Plasmid DNA has been used as a therapeutic vaccine for cancers such as melanoma, in the therapy of cardiovascular diseases, and for treatment of HIV infection.

2.4 There has been much further interest in this field for developing biomaterials, which in combination with plasmid DNA, form complexes that are better able to bind to cells and to deliver plasmid DNA into cells. When combined with cationic lipids liposome-DNA complexes are formed known as 'lipoplexes'; when combined with other biopolymeric materials, e.g., poly-L-lysine, condensed DNA complexes are formed known as 'polyplexes'. Both lipoplexes and polyplexes show significant improvements over naked plasmid DNA vectors in protection from nucleases, binding to the cell membrane, endosomal release and nuclear targeting, but are still less specific and efficient for gene delivery than viral vectors, tend to aggregate and lose efficacy, and are toxic in high concentrations.

Viral vectors

2.5 A wide range of viruses, including parvoviruses, adenoviruses, retroviruses, herpes viruses and poxviruses, is suitable for viral vector development. They are briefly described below:

Vectors derived from parvoviruses

2.6 Parvoviruses are small, single-stranded, replication-deficient DNA viruses, e.g., adeno-associated viruses (AAV)[4–6] that efficiently infect mammalian cells.AAV-2, the most widely studied serotype, is non-pathogenic, weakly immunogenic, and has a favourable safety profile. AAV-2 contains a DNA genome of 4.67 kb encoding viral capsid proteins in the *cap* gene and a 'replicase' (for DNA replication) in the *rep* gene. AAV-2 can be used to construct vectors by replacement of *rep* and *cap* sequences with a transgene, although size of the 'insert' is limited to ~4.5 kb, and *rep/cap* and additional complementing genes from adenovirus are required for propagation. Advantageously, AAV-2 particles (18–26 nm) are very robust, being stable up to 56 C and resistant to low pH and detergent, solvent, protease or nuclease attack and thus are well suited to purification by chromatographic procedures. AAV vectors can be used for *in vivo* gene delivery, e.g., to liver hepatocytes, brain, retina and skeletal muscle, with efficiencies greater than can be achieved with most other vectors; both dividing and non-dividing cells are transduced (Table 1).

2.7 Heparan sulphate proteoglycan receptors for AAV-2 are widespread making targeting of AAV-2 vectors is difficult. Use of other AAV serotypes, which bind to alternative receptors such as sialic acid-rich mucin, might offer a means for facilitating transduction of specific cell types. Once inside cells, vector single-stranded DNA is converted into a double-stranded, transcriptionally active form which, as concatamers, can persist extrachromosomally for long periods. Since the *rep* gene is absent from the vector DNA, integration into chromosomal DNA occurs only at very low levels – but this is sufficient to raise safety concerns. Parvoviral-, and in particular AAV-, vectors potentially provide very long-term transgene expression *in vivo*. Therefore, they are suitable for gene therapy of monogenic disorders such as cystic fibrosis, haemophilia B[7], α1 antitrypsin deficiency and mucopolysaccharidosis, and potentially for chronic medical conditions such as atherosclerosis and Parkinson's disease. Although to-date there have been relatively few clinical trials with AAV-vectors, interest in them is now growing with the development of better manufacturing and purification procedures.

Adenoviral vectors and conditionally replication competent adenoviruses

2.8 By comparison, Adenoviruses (Ads) are large, non-enveloped DNA viruses, e.g., human adenovirus type 5, with a linear genome of ~35 kb and thus have potential for development of vectors containing larger

transgenes.[8–10] Human Ads have a benign natural history, cause upper respiratory tract infections and, used as vaccines, have a good safety profile, except in immunocompromised individuals. They infect a broad spectrum of both quiescent and dividing cells. Ad DNA exists extrachromosomally as a 'free' linear DNA molecule during replication; integration in chromosomal DNA and the risk of insertional oncogenesis is very low.

2.9 Preparation of replication-defective, deletion mutants of human Ads, e.g. by deleting the 'early' E1 region and replacing it with heterologous DNA of up to about 8.3 kb has proved straightforward, as is their propagation to very high titres in cell lines containing complementing Ad early E1 (E1A and E1B) genes expressed *in trans*. However, such Ad vectors provoke strong host immune responses to Ad proteins, leading to vector clearance and elimination of infected cells expressing viral antigens; also most humans already have immunity to Ads which may compromise repeat dosing. In addition, systemic administration of high dose Ad vectors is associated with adverse toxicity, including acute-phase response, inflammatory reactions, systemic shock syndrome and potential lethality.[9–15]

2.10 To reduce immune and adverse responses, further modifications of Ad genomes have been studied, including deletions of early E2a (2nd generation Ad vectors), or E2a, E3 and E4 (3rd generation Ad vectors) or all early regions (4th generation Ad vectors) besides E1 deletion. Recent technical advances have made possible the development of Ad vectors devoid of all Ad genes; these are known as 'gutless' or 'high capacity' Ad vectors which can accommodate up to 32 kb of heterologous DNA.[10,14]

2.11 A further strategy to obtain tissue specificity involves development of Ads containing specific mutations, e.g., in early E1b, which, as a result, are conditionally-replication competent or 'replication-selective'. These have been designated 'conditionally-replication competent adenoviruses' (CRAds).[11] For example, in wild type Ad infections, E1b 55 kDa protein in association with adenoviral E4orf6 protein binds and inactivates the p53 tumour suppressor protein (p53), thus enabling efficient viral replication. In contrast, an E1b 55 kDa gene-mutated Ad is replication-defective in cells containing normal p53, but is able to replicate in tumour cells containing a dysfunctional p53 leading to cell death. However, a transgene encoding a therapeutic function, e.g., thymidine kinase (TK) to make tumour cells sensitive to pro-drug (ganciclovir) toxicity, may be incorporated in CRAds, in which case they may be considered as a special class of Ad vector (Table 1). Use of such CRAds in gene therapy of tumours appears attractive since infection and transgene expression in tumours lasts longer compared to replication-defective Ad vectors.[12]

2.12 Although early applications of Ad vectors focused on treatment of monogeneic diseases such as cystic fibrosis and ornithine decarbamylase (OTC) deficiency, there are safety concerns about their systemic administration at high dose levels, particularly since a young adult patient, Jesse Gelsinger, died as a direct result of Ad vector therapy of his OTC deficiency.[13] However, there have been no further reports of life-threatening toxicities found following Ad vector administration, albeit at lower doses, and there remains high interest in AdV generally, now with much more emphasis on treatment of malignancies. Ad vectors and CRAds have shown some promise in the treatment of head and neck cancers where intratumoral injections are possible. Vector shedding and spread of CRAds beyond the treated patient however remain safety concerns.

Retroviral vectors

2.13 Retroviruses are classified into seven sub-genera:- alpha-, beta-, gamma-, delta-, and epsilon-retroviruses, plus lentiviruses and spumaviruses. Gammaretroviruses, formerly known as oncoretroviruses (e.g. murine leukaemia viruses {MuLV}, gibbon ape leukaemia virus {GALV}), have been the most widely used category for vector development.[16,17] After cell entry, RNA genomes of gammaretroviruses and lentiviruses are turned into DNA copies (proviral DNA) by reverse transcriptase (RTase). Proviral DNA of

gammaretroviruses is integrated into chromosomal DNA of dividing cells only, while that of lentiviruses into both dividing and non-dividing cells. As transducing non-dividing cells could be advantageous, development of retroviral vectors has widened from gammaretroviruses to lentiviruses.[22–24]

Gammaretroviral vectors

2.14 All viral genes are removed from the viral genome between the long terminal repeat (LTR) sequences, and replaced by a transgene and its regulatory sequences of up to 6–8 kb in size. Gammaretroviral vectors (GV) based on MuLV are propagated in murine 'fibroblast' packaging cell lines with integrated 'helper-retrovirus' genes supplying viral proteins Gag, Pol and Env required for replication. GV particles can infect other (non-packaging) cells, make and integrate vector proviral DNA, but no new progeny GV can be made in target cells since they lack retroviral helper sequences.

2.15 A major safety concern for GV manufacture is that replication-competent retroviruses (RCR) may be produced by recombination events with helper viral sequences, either in packaging cells; repeated RCR infection is associated with multiple proviral DNA integrations in locations of transcriptionally-active genes with increasing risk of insertional oncogenesis and tumour development. However, additional design improvements have been made that have reduced to a minimum the risk of RCR formation. Replacing or 'pseudotyping' the homologous MuLV Env with an Env glycoprotein from another retrovirus, e.g., GALV or another family of viruses altogether, e.g., the rhabdovirus vesicular stomatitis virus (VSV), which has a broad range of domestic animals, e.g., cattle, horses and swine, as its natural hosts, can increase GV yields and facilitate infection of particular cell types, e.g., CD34+ haematopoietic stem cells[16, 18, 19].

2.16 Since GV are poorly effective *in vivo*, their main application has been transduction of human haematopoietic cells *in vitro*. For example, several male children affected by *S*evere *C*ombined *I*mmuno*d*eficiency (SCID) Syndrome secondary to a genetic deficiency of the common gamma chain (γc) of cytokine receptors, better known as X-SCID, a fatal disorder, have been successfully treated by a French group with autologously transplanted BM cells reconstituted with the normal γc gene *ex vivo* using GV transduction[20]. Unfortunately, after two/three years without complications, three children (3/11) have developed T cell leukaemias. In two cases, the cause of leukaemia has been associated with an insertion of the vector proviral DNA in or near the *LMO2* gene, which is involved in cell growth regulation and has been associated with childhood acute lymphoblastic leukaemias (ALL)[21]. Thus, this is the first clear example of insertional oncogenesis associated with GV transduction of human BM cells (see also under Section 4).

Lentiviral vectors

2.17 In comparison with gammaretroviruses the proviral DNA complex of lentiviruses has the capacity to traverse the nuclear membrane of non-dividing cells and integrate into chromosomal DNA without mitosis[22]. This is advantageous for tissues and cell populations with normally few dividing cells, e.g., in brain, eye, lung, and pancreas. Thus, lentiviral vectors (LV) may decrease the need for *ex vivo* cell manipulation and also lead to efficient gene delivery *in vivo*. However, lentiviral 'genomes' are more complex than those of the gammaretrovirus group, making design of LV a greater challenge[27]. Nevertheless, the very detailed knowledge of lentiviruses, in particular HIV-1 and -2, has made them attractive candidates for vector development.[22–24]

2.18 The main safety concerns about LV based on HIV-1 and other primate lentiviruses, e.g. SIV,[24, 25] are:- 1) potential presence of replication-competent lentiviruses (RCL) in LV preparations; 2) potential mobilisation of vector genomes by wild-type HIV-1[28]; 3) potential insertional oncogenesis. To reduce these safety risks, LV have undergone various developmental stages. Firstly, the 'virulence genes', *vpr, vpu, vif*, and *nef*, of HIV-1, were eliminated. Secondly, recombination leading to RCL formation during LV propagation has been minimised by eliminating sequence

overlaps between vector constructs. Thirdly, the HIV-1 envelope protein is replaced by VSV-G making the generation of a HIV RCL impossible. Lastly, introduction of a self-inactivating (SIN) deletion in the U3 region of the promoter in the 3¥LTR inactivates its promoter/enhancer activity, thus reducing vector mobilisation by superinfecting wild type HIV-1 and the risk of proto-oncogene activation by promoter insertion in target cells.

2.19 Nevertheless, safety concerns about primate LV remain leading manufacturers to consider non-primate lentiviruses, e.g., feline immunodeficiency virus (FIV), equine infectious anaemia virus (EIAV)[26], for LV development. Vector designs and manufacturing systems, similar to those for HIV-1-based LV, have been developed for FIV and EIAV.

2.20 To date, many clinical gene therapy protocols involving use of GV have been approved, but so far only one protocol with LV is approved. A VSV-G pseudotyped HIV-1 based LV, VRX496, incorporating a 937-base anti-sense sequence targeted to the *env* gene of HIV-1 is currently being tested in Phase I clinical trials (initiated in January 2003) involving *ex vivo* transduction of T lymphocytes from HIV-1 sero-positive patients. The idea is to protect T lymphocytes from HIV-1 infection when re-infused back into patients and thus slow the progress of the disease.

Other viral vectors

2.21 Several other viruses, including herpes viruses, poxviruses and positive- and negative-strand RNA viruses, also offer opportunities for viral vector development[1–3]. Although rapid progress has been made in their design and manufacture, they remain at the pre-clinical testing stage. Herpes viruses[29] and poxviruses[30] have high capacity for heterologous DNA incorporation, including the possibility of multiple transgenes, which could have certain advantages. The neurotoxicity of herpes viruses can be attenuated while maintaining oncolytic activity for tumour cells[31]. However, complete elimination of toxicity and immunogenicity of herpes viral vectors may not be possible, thus raising safety concerns.

2.22 In comparison, poxviruses appear more flexible; advantage can be made of attenuated poxviruses which undergo abortive replication in human cells, but maintain high transgene expression. Poxviral vectors should be suitable for *in vivo* applications, e.g., as 'cancer vaccines', as well as their more progressively acceptable use as prophylactic vaccines. Certain single-strand RNA viruses, particularly those like Semliki Forest virus that are adaptable to DNA replicon production systems, are also under development as viral vectors. They are non-integrating and achieve very high levels of transgene expression, although this tends to be transient and thus these vectors are not suitable for applications requiring long-term expression. Their major drawback is inherent cytopathogenicity.

2.23 Further progress in gene therapy will depend on either novel modifications to existing vectors that improve their efficacy and safety, or on the development of novel vector types. Modification of viral capsid or envelope proteins should allow greater specificity of gene delivery to target cells[32]. As most replication-deficient viral vectors have failed to produce a therapeutic effect, especially in cancer, replication-selective live viruses, by themselves or with incorporated transgenes, which have higher oncolytic potential are being developed and evaluated. However, virus shedding and spread to germline cells will be safety concerns for these viruses.

2.24 With regard to *ex vivo* transductions of HSC, the prospective use of LV rather than GV may improve efficacy and safety. However, extreme caution over their use will remain unless the potential risk of insertional oncogenesis can be eliminated.

References

1. Meager, A. (2003) Quality and safety of gene medicines: a practical guide. Davis-Horwood International Publishing, Goldaming, UK.

2. Hitt, M.M., and J. Gauldie. 2000. Gene vectors for cytokine expression *in vivo*. *Curr. Pharmaceut. Design.* 6:613–632.

3. Walther, W., and U. Stein. 2000. Viral vectors for gene transfer: A review of their use in the treatment of human diseases. *Drugs.* 60:249–271.

4. Flotte, T.R. and B.J. Carter. 1999. Adeno-associated viral vectors. In *Gene therapy technologies, applications and regulations: from laboratory to clinic*, edited by A. Meager. Chichester: John Wiley.

5. Tal, J. 2000. Adeno-associated virus-based vectors in gene therapy. *J. Biomed. Sci.* 7:279–291.

6. Ponnazhagan, S., D.T. Curiel, D.R. Shaw, et al. 2001. Adeno-associated virus for gene therapy. *Cancer Res.* 61:6313–6321.

7. Kay, M.A., C.S. Manno, M.V. Ragni, et al. 2000. Evidence for gene transfer and expression of Factor IX in hemophilia B patients treated with an AAV vector. *Nature Genetics.* 24:257–261.

8. Connelly, S. 1999. Adenoviral vectors. In *Gene therapy technologies, applications and regulations: from laboratory to clinic*, edited by A. Meager. Chichester: John Wiley.

9. Brenner, M. 1999. Gene transfer by adenoviruses. *Blood.* 94:3965–3967

10. Morsy, M.A., and C.T. Caskey. 1999. Expanded-capacity adenoviral vectors- the helper-dependent vectors. *Mol Med Today.* 5:18–24.

11. Kruyt, F.A.E., and D.T. Curiel. 2002. Toward a new generation of conditionally replicating adenoviruses: pairing tumor selectivity with maximal oncolysis. *Hum. Gene Ther.* 13:485–495.

12. Wildner, O., J.C. Morris, N.N. Vahanian, et al. 1999. Adenoviral vectors capable of replication improve the efficacy of HSVtk/GCV suicide gene therapy of cancer. *Gene Ther.* 6:57–62.

13. NIH Report. 2002. Assessment of adenoviral vector safety and toxicity: Report of the National Institutes of Health Recombinant DNA Advisory Committee. *Hum. Gene Ther.* 13:3–13.

14. Kochanek, S. 1999. High-capacity adenoviral vectors for gene transfer and somatic gene therapy. *Hum. Gene Ther.* 10:2461–2469.

15. Morral, N., W.K. O'Neal, K. Rice, et al. 2002. Lethal toxicity, severe endothelial injury, and a threshold effect with high doses of an adenoviral vector in baboons. *Hum. Gene Ther.* 13:143–154.

16. Yee, J.K. 2000. Retroviral vectors. In *The Development of Human Gene Therapy*, edited by T. Friedmann. Cold Spring Harbor, NY: Cold Spring Habor Laboratory Press.

17. Günzburg, W.H. and B. Salmons. 1999. Retoviral vectors. In *Gene therapy technologies, applications and regulations: from laboratory to clinic*, edited by A. Meager. Chichester: John Wiley.

18. Friedmann, T., and J.K. Yee. 1995. Pseudotyped retroviral vectors for studies of human gene therapy. *Nature Med.* 1:275–277.

19. Kiem, H.P., S. Heyward, and A. Winkler. 1997. Gene transfer into marrow repopulating cells: comparison between amphotropic and gibbon ape leukemia virus pseudotyped retroviral vectors in a competitive repopulation assay in baboons. *Blood.* 90:4638–4645.

20. Cavazzana-Calvo, M., S. Hacien-Bey, F. Yates, et al. 2001. Gene therapy of severe combined immunodeficiencies. *J. Gene Med.* 3:201–206.

21. Kaiser, J. 2003. Seeking the cause of induced leukemias in X-SCID trial. *Science* (Wash.) 299:495.

22. Lever, A.M.L. 1999. Lentiviral vectors. In *Gene therapy technologies, applications and regulations: from laboratory to clinic*, edited by A. Meager. Chichester: John Wiley.

23. Vigna, E., and L. Naldini. 2000. Lentiviral vectors: excellent tools for experimental gene transfer and promising candidates for gene therapy. *J. Gene Medicine.* 2:308–316.

24. Buchschacher, G.L.Jr., and F. Wong-Staal. 2000. Development of lentiviral vectors for gene therapy for human diseases. *Blood.* 95:2499–2504.

25. White, S.M., M. Renda, N.Y. Nam, et al. 1999. Lentivirus vectors using human and simian immunodeficiency virus elements. *J. Virology.* 73:2832–2840.

26. Mitrophanous, K., S. Yoon, J. Rohll, et al. 1999. Stable gene transfer to the nervous system using a non-primate lentiviral vector. *Gene Ther.* 6:1808–1818.

27. Kumar, M., B. Keller, N. Makalou, and R.E. Sutton. 2001. Systematic determination of the packaging limit of lentiviral vectors. *Hum. Gene Ther.* 12:1893–1905.

28. Evans, J.T. and J.V. Garcia. 2000. Lentivirus vector mobilization and spread by human

immunodeficiency virus. *Hum. Gene Ther.* 11:2331–2339.

29. Soares, M.K., W.F. Goins, J.C. Glorioso, and D.J. Fink. 1999. Advances in engineering HSV vectors for gene transfer to the nervous system. In *Gene therapy technologies, applications and regulations: from laboratory to clinic*, edited by A. Meager. Chichester: John Wiley.

30. Mastrangelo, M.J., L.C. Eisenlohr, L. Gomella, and E.C. Lattime. 2000. Poxvirus vectors: orphaned and underappreciated. J. Clin. Invest. 105:1031–1034.

31. Martuza, R. 2000. Conditionally replicating herpes virus for cancer therapy. J. Clin. Invest. 105:841–846.

32. Wickham, T.J. 2000. Targeting adenovirus. *Gene Ther.* 7:110–114.

Appendix 1- *Gene therapy of inherited and acquired diseases*

A. Inherited monogeneic diseases

Of 987 clinical gene therapy trials worldwide, 9.4% have been for monogeneic diseases, and all of these have been Phase I, or Phase I/II (Wiley website).

Gene therapy for primary haematological/immunological disorders

The accessibility of the haematopoietic system either through bone marrow progenitors or mature cells has made this group of diseases a highly favourable target for gene therapy. The ability of haematopoietic stem cells (HSC) to self renew and give rise to all blood lineages (as demonstrated in more conventional allogeneic stem cell transplantation) adds significant potency as a vehicle for curative therapy of these diseases. For instance, in the severest forms of primary immunodeficiency, known as severe combined immunodeficiencies (SCIDs), where T lymphocyte development is invariably interrupted, gene transfer need only impart a survival or growth advantage to HSC. Recently 4 clinical trials have demonstrated sustained therapeutic efficacy for X-linked SCID and adenosine deaminase-deficient SCID (ADA-SCID). Advances in gene transfer technology, e.g., activation of cells with specific cytokines (thereby making them more susceptible to gammaretrovirus vector-mediated gene transfer), and patient preparation have contributed to these successes.

Treatment of X-linked SCID

X-linked SCID (SCID-X1) accounts for approximately 50–60% of all SCIDs, and is caused by mutations in the gene encoding the common cytokine receptor gamma chain (γc), a subunit of receptor complexes for interleukins (IL) 2, 4, 7, 9, 15 and 21. Without γc-signaling, immune cell development and function are compromised. If a genotypically matched family donor is available, bone marrow transplantation is a highly successful procedure with a long-term survival rate of over 90% (Antoine et al, 2003). For most patients, this is not possible, and the survival from mismatched family (usually parental donors) transplants is lower, and associated with predictable toxicity from the use of alkylating agents used to enhance engraftment. Two studies have now demonstrated efficacy of treatment using autologous HSC transduced *ex vivo* to express γc, either by an amphotropic or a GALV-pseudotyped gammaretroviral vector (Cavazzana-Calvo et al, 2000; Hacein-Bey et al, 2002; Gaspar et al, in press; Table 2).

Presently, 16 infants (in addition to one adult with failing immunity following allogeneic transplantation in infancy, and one child with atypical disease and late presentation) have been so treated; 15 have achieved substantial and sustained immunological correction. (Development of serious adverse events in two of these will be discussed below). These outcomes compare extremely favourably with a predicted 15% mortality associated with mismatched transplantation, and inevitable morbidity arising from administration of chemotherapy (Antoine et al, 2003). The longevity of functional reconstitution, determined by clinical monitoring, remains unknown, but it may be possible to repeat gene therapy. However, two attempts to treat an adult patient with persisting immunodeficiency after allogeneic transplantation in infancy and an older child with incomplete γc-deficiency have failed using identical protocols, suggesting that there may be age-related restrictions to (re)initiation of thymopoiesis (Thrasher, Hacein-Bey-Abia et al, submitted).

Treatment of ADA-deficient SCID

Deficiency of the purine salvage enzyme adenosine deaminase (ADA) accounts for approximately 10–20% of all SCIDs (ADA-SCID). ADA catalyses the deamination of deoxyadenosine (dAdo) and adenosine to deoxyinosine and inosine respectively and the lack of ADA leads to the build of the metabolites deoxyATP (dATP) and dAdo which have toxic effects on lymphocyte development and function, leading to minimal numbers of T and B lymphocytes. Bone marrow transplantation is highly successful in the genotypically-matched setting, but human leucocyte antigen (HLA)-mismatched transplants have poor survival outcomes. Alternatively, intramuscular injections of

Disease	Gene	Retroviral vector	Envelope	Cell type treated/ No. of patients	Reference/PI
ADA-D	ADA	GIADA1	A	*BM $CD34^+$ (5)	Aiuti et al, 2002
ADA-D	ADA	GCsap-M-ADA MND-ADA	A	BM $CD34^+$ (4) UCB $CD34^+$ (3)	D Kohn and F Candotti, Schmidt et al, 2003
ADA-D	ADA	SFFV-ADA-WPRE	G	*BM $CD34^+$ (1)	AJ Thrasher/ HB Gaspar
ADA-D	ADA	?	A	BM $CD34^+$ (?1)	? Y Sakiyama
SCID-X1	gc	MFG	A	BM $CD34^+$ (11)	Cavazzana-Calvo et al, 2000; Hacein Bey Abina, 2002)
SCID-X1	gc	MFG	G	BM $CD34^+$ (7)	Gaspar et al, in press
SCID-X1	gc	MFG	G	PB $CD34^+$ (1)	J Puck, H Malech
AR-SCID	JAK-3	MSCV	G	BM $CD34^+$ (1)	BP Sorrentino
X-CGD	Gp91phox	SF71gp91phox	G	*BM/PB $CD34^+$ (212)**	M Grez A Thrasher R Seger

ABBREVIATIONS:

A, amphotropic envelope
G, gibbon ape leukaemia virus envelope
ADA, adenosine deaminase deficiency
SCID-X1; X-linked SCID
X-CGD; X-linked CGD
BM, bone Marrow
UCB, umbilical cord blood
PB; mobilised peripheral blood
γc, common cytokine receptor gamma chain

* Denotes the use of pre-conditioning.
** including 2 patients treated with MFG-gp91phox and SFgp91phox-LNGFR.

polyethylene glycol-conjugated bovine ADA can result in correction of metabolic and immunological abnormalities, but are often only partially effective.

Matched sibling donor transplants for ADA-SCID performed without conditioning result in rapid engraftment and persistence of donor T lymphocytes, indicating that T lymphocytes expressing ADA have a powerful proliferative and survival advantage. Clinical gene transfer studies in patients with ADA deficiency were initiated in the early 1990's and have continued through to the present. Autologous T lymphocytes or CD34+ stem cells were transduced *ex vivo* with gammaretroviral vectors harbouring the ADA gene and then reinfused into patients. Although these were therapeutically ineffective, analysis of patient samples has demonstrated persistence of transduced T cells and sustained transgene expression (Muul et al, 2002; Schmidt et al, 2003). Failure of proliferation and expansion of transduced T cells most likely resulted from the concurrent administration of PEG-ADA, which abrogated this growth advantage.

Withdrawal of PEG-ADA from one patient participating in another study demonstrated convincingly that a survival advantage exists for gene-transduced cells (Aiuti et al, 2002a). In two recent studies, in which 4 patients received mild conditioning but no PEG-ADA, there are clear

indications that ADA-SCID can be successfully treated with amphotropic gammaretroviral vector transduced CD34+ cells expressing ADA (Aiuti et al, 2002b; Gaspar and Thrasher, unpublished observations; Table 2). Sustained restoration of immunity has been observed in all patients suggesting that multipotent progenitors have engrafted. These encouraging signs are leading to further studies, e.g., using a GALV-pseudotyped gammaretroviral vector incorporating regulatory sequences from the Spleen Focus Forming Virus LTR and a Woodchuck post-transcriptional regulatory element to maximise ADA expression (Gaspar and Thrasher, unpublished) for *ex vivo* transductions.

Gene therapy for other haematological and immunological disorders

Similar clinical gene transfer approaches to those for treating of SCID-X1 and ADA-SCID appear potentially useful for treating other monogeneic haematological and immunological disorders. For example, mutation in the *JAK-3* gene, encoding a tyrosine kinase for interleukin receptor signaling, is responsible for a clinical and immunological phenotype identical to that of SCID-X1 . Thus, the rationale for gene therapy is similar, and has been successfully implemented in a murine model of JAK-3 deficient SCID (Bunting et al, 2000). Patients with mutations of the recombinase activating genes *RAG-1* and *RAG-2* characteristically present with absence of both B and T cells; again effective reconstitution has been shown in RAG-2 deficient mice using a similar gene therapy protocol (Yates et al, 2002). Patients with Wiskott-Aldrich Syndrome or Fanconi anaemia appear also to be good candidates for this type of clinical gene transfer where a growth and survival advantage is conferred to biochemically corrected cells.

For many other diseases, including the common haemaglobinopathies (thallassaemia and sickle cell anaemia), difficulties in achieving efficient gene transfer and long-term engraftment of functionally corrected cells remain. Chronic Granulomatous Disease (CGD) is a primary immunodeficiency caused by defects in a phagocyte enzyme NADPH-oxidase complex, and essentially a paradigm for this situation. No growth advantage is conferred to transduced cells, and gene expression is important only in terminally differentiated granulocytes and macrophages. Early studies in which amphotropic gammaretroviral vector transduced CD34+ cells were used reported only transient evidence for functional activity in circulating neutrophils (Malech, 1999), although prior low-intensity conditioning can result in sustained production of functionally corrected cells (unpublished observations). For diseases such as CGD, correction of 100% of cells unnecessary, although the levels of correction on a per cell basis may be limiting.

Gene therapy for haemophilia

Animal models (mice and dogs) of haemophilia have shown, using gene therapy approaches focused on AAV vectors, that partial restoration (1–2% increase in levels) of clotting activity will be therapeutically useful. Subsequently, two clinical trials for haemophilia B (Factor IX (FIX)-deficiency) have been performed with AAV2 vectors. The first was a dose escalation study of intramuscular injection in 8 patients, and although FIX was detectable immunohistochemically at the injection site, only transient sub-therapeutic levels were achieved systemically (Manno et al, 2003). More recently, a second study has been suspended following immune responses to AAV2 capsids, which resulted in transient mild hepatitis and coincident elimination of detectable FIX transgene expression in 2 patients treated by intra-hepatic artery infusions (Katherine High, ASGT 2004). Development of alternative AAV serotypes coupled with immunosuppressive protocols at the time of therapy may achieve efficacy. There is an urgent need for animal models that more closely mimic the human response, although current findings highlight the importance of human clinical trials for evaluation and refinement of new therapies.

Gene therapy studies for non-haematological monogeneic disorders

Cystic fibrosis is an autosomal recessive multisystem disorder of the CF transmembrane regulator gene (CFTR), which is the commonest cause of severe lung disease and exocrine pancreatic insufficiency in childhood (approximately 1/2500 children born in UK). The first gene therapy trials were conducted in 1993, and at least 18 have subsequently been initiated (Griesenbach et al, 2002). Both viral (Ad and AAV2) and non-viral systems have been applied to either

nasal epithelium or lower airways, and although gene transfer has been demonstrable, efficiency has been too low for clinical benefit.

Particularly important are the difficulties in reaching appropriate target cells *in vivo* after topical airway application, and also the need for suppression of immunological responses (particularly relevant for viral vectors). Similarly, clinical gene therapy for inherited muscle diseases, e.g., Duchenne Muscular Dystrophy (DMD), has been limited by inefficient gene delivery. Recently, widespread transduction of cardiac and skeletal muscle has been achieved in an animal model following a single intravenous injection of AAV6 vector in combination with vascular endothelium growth factor (VEGF) (Gregorivic et al, 2004), which induces acute permeabilization of peripheral microvasculature. This procedure, which appears to enhance efficiency of transduction, may be applicable to a wide range of muscular disorders.

Other diseases likely to become targets for gene therapy include inherited retinal degeneration, for which particularly encouraging pre-clinical data have been achieved (Bessant et al 2001). Novel therapeutic strategies are being investigated, including administration of recombinant neurotrophic factors, photoreceptor-, retinal pigment epithelium (RPE)-specific or stem cell transplantation and, significantly, gene therapy. Efficient *in vivo* gene transfer to photoreceptor cells and the RPE following subretinal injection of AAV or lentiviral vectors with cell-specific promoters has been demonstrated in a rodents, dogs and primates with minimal inflammation and toxicity (Bennett et al 1999; Dudus et al 1999; Sarra et al 2002; Bainbridge et al 2003). Recent studies have also shown either retardation of photoreceptor cell loss or functional improvement in animal models of retinal degeneration (McGee et al 2001; Liang et al 2001; Acland et al 2001; Ali et al 2000). Clinical trials for these conditions appear imminent.

Safety issues for gene therapy of inherited monogeneic diseases

For retroviruses, which depend on chromosomal integration for stability of transduction, the most prominent safety concern has been for insertional oncogenicity. On the basis of numerous animal studies and over 300 clinical trials in which patients have received retroviral vectors, and from theoretical considerations, the risk of clinically manifesting malignancies due to insertional mutagenesis has been judged to be low (transformation frequency of 10^{-7} determined in growth factor-dependent cell lines *in vitro*) (also see report from ASGT ad hoc committee on retrovirus gene transfer to HSC, http://www.asgt.org). However, several studies have shown gammaretroviral vectors have a preference for insertion close to the promoter region of active genes, indicating that risks due to insertional mutagenesis may be skewed (Wu et al, 2003; Baum et al, 2004).

Three of 11 patients with SCID-XI treated with gammaretroviral vector-transduced stem cells have developed lymphoproliferative disease 2–3 years after the gene therapy procedure (Hacein-Bey-Abina, 2003). In two patients, proviral DNA insertion into or near the *LMO-2* protooncogene resulted in high-level expression of LMO-2, which is known to participate in human leukaemogenesis by chromosomal translocation and, in mice, results in latent development of T cell lymphoproliferation and leukaemia. It is likely that other contributing factors, e.g., the very young age of the patients treated, possibly dysregulated γc expression in lymphoid cells, are required for these adverse events (Dave et al, 2004). The integration of the vector proviral DNA into *LMO-2* in both cases strongly suggests that there is some preference for survival of these clones or less likely, for integration at this site (Hacein-Bey-Abina et al. 2003). Detailed molecular analysis of vector insertions the genome of patients undergoing such gene therapy will greatly assist in the delineation of integration points, but is unlikely to predict potential for oncogenesis unless recurrent hotspots associated with clinical disease become evident (Schroder et al, 2002; Laufs et al, 2002).

Strategies to avoid or reduce oncogenesis, e.g., by modifications to the intrinsic design of vectors, are therefore of key importance. The use of 'insulators' or self-inactivating (SIN) mutations in retroviral vectors may help reduce the risk of oncogenesis (Burgess-Beusse et al, 2002; Olivares et al, 2002). Differences in patterns of integration may also influence safety. For example, lentiviral vectors share a preference for integration at active gene sites, but are less targeted to promoter regions than gammaretroviral vectors (Schroder et al, 2002). SIN lentiviral vectors also appear to provide greater capacity for incorporation of more complex and physiological regulatory sequences, but any safety

advantage of such modifications needs to be determined in clinically relevant animal-model systems and the effectiveness of these models to predict side effects in humans will have to be validated. Methods to control the number of integration events per cell and to limit the number of engrafting clones may also be beneficial (at present the dose of transduced cells that is required for clinical benefit is undetermined).

References

Acland, G.M., G.D.Aguirre, J.Ray, *et al.* 2001. Gene therapy restores vision in a canine model of childhood blindness. *Nat.Genet.* 28:92–95.

Aiuti, A., S.Vai, A.Mortellaro, *et al.* 2002(a). Immune reconstitution in ADA-SCID after PBL gene therapy and discontinuation of enzyme replacement. *Nat.Med.* 8:423–425.

Aiuti, A., S.Slavin, M.Aker, *et al.* 2002(b). Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. *Science* 296:2410–2413.

Ali, R.R., G.M.Sarra, C.Stephens, *et al.* 2000. Restoration of photoreceptor ultrastructure and function in retinal degeneration slow mice by gene therapy. *Nat.Genet.* 25:306–310.

Antoine, C., S.Muller, A.Cant, *et al.* 2003. Long-term survival and transplantation of haemopoietic stem cells for immunodeficiencies: report of the European experience 1968–99. *Lancet* 361:553–560.

Bainbridge JBL, Mistry A, Thrasher A, and Ali RR. 2003. Gene therapy for ocular angiogenesis. *Clinical Science* 104:561–575.

Baum, C., C.von Kalle, F.J.Staal, *et al.* 2004. Chance or necessity? Insertional mutagenesis in gene therapy and its consequences. *Mol Ther.* 9:5–13.

Bennett J, A.M.Maguire, A.V.Cideciyan, *et al.* 1999. Stable transgene expression in rod photoreceptors after recombinant adeno-associated virus-mediated gene transfer to monkey retina. *Proc Natl Acad Sci U S A* 96: 9920–5.

Bessant, D.A., R.R.Ali, and S.S.Bhattacharya. 2001. Molecular genetics and prospects for therapy of the inherited retinal dystrophies. *Curr.Opin.Genet. Dev.* 11:307–316.

Bunting, K.D., T.Lu, P.F.Kelly, and B.P.Sorrentino. 2000. Self-selection by genetically modified committed lymphocyte precursors reverses the phenotype of JAK3-deficient mice without myeloablation. *Hum.Gene Ther.* 11:2353–2364.

Burgess-Beusse,B., C.Farrell, M.Gaszner, *et al.* 2002. The insulation of genes from external enhancers and silencing chromatin. *Proc.Natl.Acad Sci.U.S.A* 99 Suppl 4:16433–16437.

Cavazzana-Calvo,M., S.Hacein-Bey, B.G.de Saint, *et al.* 2000. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science* 288:669–672.

Dave,U.P., N.A.Jenkins, and N.G.Copeland. 2004. Gene therapy insertional mutagenesis insights. *Science* 303:333.

Dudus L, V.Anand, G.M.Acland, *et al.* 1999 Persistent transgene product in retina, optic nerve and brain after intraocular injection of rAAV. Vision Res 39: 2545–53

Gaspar HB, Parsley KL, Howe S, *et al.* 2004. Successful gene therapy of SCID-X1 using a pseudotyped gammaretroviral vector. *Lancet* In press.

Griesenbach,U., S.Ferrari, D.M.Geddes, and E.W.Alton. 2002. Gene therapy progress and prospects: cystic fibrosis. *Gene Ther.* 9:1344–1350.

Gregorevic, P., M.J.Blankinship, J.M. Allen, *et al.* 2004. Systemic delivery of genes to striated muscles using adeno-associated viral vectors. *Nat.Med* Online pre-publication.

Hacein-Bey-Abina,S., F.Le Deist, F.Carlier, *et al.* 2002. Sustained correction of X-linked severe combined immunodeficiency by ex vivo gene therapy. *N.Engl.J Med* 346:1185–1193.

Hacein-Bey-Abina,S., C.von Kalle, M.Schmidt, *et al.* 2003. LMO2-Associated Clonal T Cell Proliferation in Two Patients after Gene Therapy for SCID-X1. *Science* 302, 415–419.

Laufs,S., B.Gentner, K.Z.Nagy, *et al.2002.* Retroviral vector integration occurs into preferred genomic targets of human bone marrow repopulating cells. *Blood* 101:2191–2198.

Liang FQ, T.S.Aleman, N.S. Dejneka, *et al.* 2001 Long-term protection of retinal structure but not function using RAAV.CNTF in animal models of retinitis pigmentosa. Mol Ther 4: 461–72

Malech,H.L. 1999. Progress in gene therapy for chronic granulomatous disease. *J Infect.Dis* 179 Suppl 2:S318–S325.

Manno,C.S., A.J.Chew, S.Hutchison, *et al.* 2003. AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B. *Blood* 101:2963–2972.

McGee L, L.H. Sanftner, H.Abel, *et al.* 2001 Glial cell line derived neurotrophic factor delays photoreceptor degeneration in a transgenic rat model of retinitis pigmentosa. Mol Ther 4: 622–9.

Muul,L.M., L.M.Tuschong, S.L.Soenen, *et al.* 2002. Persistence and expression of the adenosine deaminase gene for twelve years and immune reaction to gene transfer components: Long-term results of the first clinical gene therapy trial. *Blood* 101: 2563–2569.

Olivares,E.C., R.P.Hollis, T.W.Chalberg, *et al.* 2002. Site-specific genomic integration produces therapeutic Factor IX levels in mice. *Nat.Biotechnol.* 20:1124–1128.

Sarra,G.M., C.Stephens, F.C.Schlichtenbrede, *et al.* 2002. Kinetics of transgene expression in mouse retina following sub-retinal injection of recombinant adeno-associated virus. *Vision Res.* 42:541–549.

Schmidt,M., D.A.Carbonaro, C.Speckmann, *et al.* 2003. Clonality analysis after retroviral-mediated gene transfer to CD34+ cells from the cord blood of ADA-deficient SCID neonates. *Nat.Med* 9:463–468.

Schroder,A.R., P.Shinn, H.Chen, *et al.* 2002. HIV-1 integration in the human genome favors active genes and local hotspots. *Cell* 110:521–529.

Thrasher,A.J., S.Hacein-Bey-Abina, H.B.Gaspar, *et al.* 2004. Failure of SCID-X1 gene therapy in older patients. *Submitted to Blood.*

Wu, X., Y.Li, B.Crise and S.M. Burgess. 2003. Transcription start regions in the human genome are favored targets for MLV integration. *Science* 300:1749–1751.

Yates,F., M.Malassis-Seris, D.Stockholm, *et al.* 2002. Gene therapy of RAG-2-/- mice: sustained correction of the immunodeficiency. *Blood* 100:3942–3949.

B. Cardiovascular Diseases

Introduction

Acquired cardiovascular diseases remain major causes of morbidity and mortality in modern societies. For acquired cardiovascular diseases of known pathology several clinical therapies are possible. Surgical interventions are often curative treatments for cardiopathologies, but they may induce undesirable effects, e.g. fibrosis, restenosis, which require additional therapy. While contemporary treatments are reducing the cardiovascular disease burden, there remains an urgent need to explore novel therapeutic strategies. Greater understanding of molecular pathophysiological processes underlying cardiovascular syndromes has opened possibilities for research and clinical studies using sophisticated therapeutic techniques, including the transfer of genes encoding therapeutic proteins to somatic cells by gene therapy. In this regard, gene therapy applications to cardiovascular disease were among the earliest in this field due to availability of surgical techniques and percutaneous catheters for anatomical access to the compartmentalized structure of the heart (see Kizana & Alexander, 2003 for Review). They have continued through the last decade as the means for gene transfer, cell targeting and control of transgene expression improve.

Genes may be readily delivered to specific heart sites using double balloon catheters, which afford protection from proximal and distal spread of vectors. Nevertheless, efficiency of gene transfer and proportion of gene-modified cells required for syndrome/symptom correction are crucial factors for success. For instance, augmentation of heart contractibility in heart failure will depend on gene-modification of a large proportion of cardiomyocytes. In contrast, targeting coronary vasculature for correction of vasoproliferative diseases may be accomplished by focal gene transfer to relatively small blood vessel segments. The availability of a wide range of vectors should make it possible to match specific vector types to medical purpose.

Cardiovascular disease targets and potential gene therapy interventions

Currently, the main focus in cardiovascular disease is on induction of angiogenesis to promote the growth of new blood vessels and improve blood supply in ischaemic tissues using angiogenesis-promoting genes, such as fibroblast growth factors (FGF) and vascular endothelial growth factors (VEGF). However, other cardiac diseases involving multiple clinical syndromes can be considered as potential gene therapy targets as well as undesirable sequelae, e.g., restenosis, that follow surgical procedures. Much pre-clinical research has been conducted in animal model systems of heart failure, myocardial ischaemia, restenosis, vascular thrombosis, focal arrhythmia and abnormal repolarisation (Table 3). Identification of regulatory proteins and enzymes underlying particular physiological or pathophysiological processes has promoted investigations into the potential value of gene transfer methods for modulating and correcting relevant molecular processes that could lead eventually to clinical benefits. Experimental strategies, corrective genes for transfer, delivery techniques and most potentially useful vector systems for cardiac disease are listed in Table 3 (arrows indicate beneficial up- or down-regulation).

Heart failure

Heart failure is the most common clinical syndrome of cardiac disease, irrespective of etiology, with a prognosis similar to many advanced cancers. As treatment options are often limited to heart transplantation, itself limited by donor shortages, and mechanical ventricular assist devices, management and treatment of late stage heart failure patients is difficult. Interim therapies, including gene transfer approaches, e.g., to delay further deterioration until a suitable cardiac allograft becomes available, are increasingly important. For example, the beta-adrenergic receptor (BAR) signaling cascade is a major modulator of normal cardiac function. Therefore, transfer of genes encoding proteins involved in the BAR signaling cascade (Table 3) should increase contractility, chronotropy (heart rate) and dromotropy (cardiac conduction). For example, *in vivo* adenoviral vector-mediated gene transfer of adenylyl cyclase by intracoronary delivery to porcine hearts has recently been reported to result in improved BAR agonist-stimulated hemodynamics (Lai et al., 2000). Although the true value of augmenting adrenergic signaling in heart failure is not fully understood, such promising gene therapy

Targets	Strategy	Transgenes	Delivery	Vector system
Heart failure	Augment β-adrenergic receptor (BAR) signaling	BAR↑; G-protein↑; Adenylyl cyclase↑; BAR kinase (BARK)↓	Intracoronary; Transcoronary; *Ex vivo* venous retroinfusion	AAV; Lentiviral
	Improve calcium handling	Sarcoplasmic reticulumCaATPase (SERCA)↑; phospholamban ↓		
	Inhibit apoptosis	Bcl-2 ↑; PI-3 kinase ↑; Atk ↑; caspase-3 inhibitor p35 ↑ NF-KB ↓; IL-1ra ↑;		
	Inhibit inflammation	IL-10 ↑		
	Inhibit oxidant stress	MnSOD↑; catalase↑; heme oxygenase-1 (HO-1)↑		
	Cardiomyoplasty	Myogenic transcription factor MyoD↑; VEGF (*ex vivo*) ↑; CTLA4-Ig↑		
Myocardial ischemia	Induce angiogenesis	FGF-5↑; $VEGF_{165}$↑; $VEGF_{121}$↑	Intracoronary; Endicardial; Epicardial; Venous retroinfusion	Non-viral plasmid; Adenoviral

Restenosis	Prodrug dependent cytotoxicity	HSV-thymidine kinase (HSV-tk) ↑;	Intracoronary	Non-viral plasmid; Adenoviral
	Cell cycle modulation	Retinoblastoma gene (Rb) ↑; cyclase-dependent kinase inhibitors p21 and p27↑		
	Inhibit cell migration	Tissue inhibitors of matrix metalloproteins (TIMPs) ↑		
	Accelerate re-endothelisation	VEGF↑; nitric oxide (NO) ↑; prostacyclin		
	Inhibit thrombosis	Hirudin↑		
Vascular thrombosis	Inhibit coagulation	Hirudin↑; thrombomodulin↑; tissue-factor pathway inhibitor (TFPI) ■; cyclooxygenase-1↑; NO synthase	Intracoronary	Non-viral plasmid; Adenoviral
	Promote fibrinolysis	Recombinant tissue-type plasminogen activator (rTPA) ↑		
	Enhance endothelial function	VEGF↑		
Focal arrythmia	Augment adrenergic sensitivity	B_2AR↑	Selective intracoronary (AV mode); Endocardial	AAV; Lentiviral
	Induce diastolic depolarizastion	Rectifier current inhibitor (Ik_1I) ↑		
	Modulate conduction	Inhibitory G-protein (G_{ai2}) ↑		
Repolarisation abnormality	Reduce action potential duration	Rectifier current ion channel proteins, e.g., a subunit of Ik_s, Kir2.1, KCNE3↑	Intracoronary; Transcoronary; *Ex vivo* venous retroinfusion	AAV; Lentiviral

approaches may well reach the clinic in the near future. Other studies with alternative targets, e.g., calcium handling proteins, or involving experimental approaches to prevent cellular demise, e.g., anti-apoptotic, anti-oxidant approaches, are also in progress (Table 3).

Therapeutic angiogenesis

Atherosclerosis is the most prevalent pathological process affecting human adult coronary arteries. To unblock arteries, patients usually need re-vascularization in the form of either balloon angioplasty and stenting or coronary bypass grafting. While most patients benefit from these invasive treatments, a significant subset continue to have myocardial ischemia and represent a target for a gene therapy strategy, known generically as 'therapeutic angiogenesis'.

The object of therapeutic angiogenesis is to use angiogenic factors to induce angiogenesis and improve perfusion of ischemic muscle and tissues. Much progress has been made with therapeutic angiogenesis in experimental studies. Favourably, gene delivery can be made using simple and safe gene transfer vehicles, such as DNA plasmids, lipoplexes or polyplexes. Alternatively, adenoviral vector-mediated transient transgene expression is sufficient to meet requirements for inducing therapeutic angiogenesis. Perhaps, the most notable pre-clinical study of myocardial ischemia is the porcine model of stress-induced myocardial ischemia, in which catheter-effected adenoviral vector-mediated transfer of FGF-5 resulted in improved blood supply (Giordiano et al., 1996). In addition, both plasmid-mediated- and adenoviral vector-mediated- VEGF gene transfer have been demonstrated to result in significant angiogenesis (Tio et al., 1999; Mack et al., 1998).

Improved tissue perfusion of previously ischemic myocardium and subjective benefits in the form of reduced symptom load have been reported in patients with refractory myocardial ischemia following DNA-plasmid-mediated $VEGF_{165}$ gene transfer effected by direct trans-epicardial injection during minithoracotomy (Losordo et al., 1998; Symes et al., 1999; Vale et al., 2000). An approach in follow-up trials using a catheter-based trans-endocardial delivery of plasmid-VEGF has been reported to achieve both subjective and objective improvements when compared to injection of a placebo.

Concurrently with plasmid-mediated protocols, phase I clinical trials involving administration of adenoviral vectors with either $VEGF_{121}$ or FGF-5 to patients with refractory myocardial ischemia have taken place. Some improvements in symptoms were recorded in the patients treated with AdVEGF (Rosengart et al 1999), while some complications and deaths, with causes probably unrelated to treatment, occurred in the patients receiving AdFGF (Grines et al., 2002). Some safety concerns attend the use of such vectors, including development of neutralizing adenoviral antibodies, adenovirus provoked thrombus formation (LaFont et al., 1998) and the potential of angiogenic inducing factors to promote and accelerate tumour growth.

In addition, similar therapeutic angiogenesis approaches are being developed for the treatment of peripheral vascular disease and critical limb ischemia (CLI) (Isner et al., 1996; Baumgartner et al., 1998). Patients in two clinical trials involving plasmid-mediated $VEGF_{165}$ gene transfer by intra-arterial or intramuscular delivery have shown symptomatic benefits, including relief of pain, healing of ulcers, and objective improvements such as formation of collateral vessels. Adenoviral vector- and plasmid/liposome-mediated VEGF gene delivery into ischemic legs during angioplasty has improved vascularity of treated limbs three months after commencement of treatment (Mäkinen et al., 2002).

Besides VEGFs and FGFs, there are known to be several other angiogenesis-inducing factors. One of these factors, hypoxia-inducible factor-1α (HIF-1α), is a transcriptional regulatory factor that, in combination with its constitutively expressed dimerization partner, HIF-1β, plays a principal role in cellular responses to changes in oxygen tension. HIF-1α is induced by low oxygen tension as occurs in ischemia; it transcriptionally activates a number of genes encoding angiogenesis-inducing proteins, including VEGF isoforms, angiopoietins 2 and 4, platelet-derived growth factor (PDGF), and placental growth factor (PlGF). HIF-1α gene delivery in pre-clinical ischemic model systems, e.g., rabbit ischemic hindlimb, has been shown to result in significant improvement in perfusion (increased blood supply and improved blood pressure ratio). Clinical trials

using adenoviral vector-mediated HIF-1α (Ad2/HIF-1α) delivery in patients with advanced coronary artery disease or CLI are currently in progress. The best sign of bioactivity has been complete ulcer healing, with an apparent trend in higher doses of vector.

References

Baumgartner, I., Pieczek, A., Manor, O., et al. 1998. Constitutive expression of phVEGF165 after intramuscular gene transferpromotes collateral vessel development in patients with critical limb ischemia. Circulation 97:1114–23.

Giordiano, F.J., Ping, P., McKirnan, M.D., et al. 1996. Intracoronary gene transfer of fibroblast growth factor-5 increases blood flow and contractile function in an ischemic region of the heart. Nat. Med. 2:534–39.

Grines, C.L., Watkins, M.W., Helmer, G., et al. 2002. Angiogenic gene therapy (AGENT) trial in patients with stable angina pectoris. Circulation 105:1291–7.

Hedman, M., Hartikainen, J., Syvänne, M., et al. 2003. Safety and feasibility of catheter-based local intracoronary vascular endothelial growth factor gene transfer in the prevention of postangioplasty and instent restenosis and in the treatment of chronic myocardial ischemia. Circulation 107:2677–83.

Isner, J.M., Pieczek, A., Schainfeld, R., et al. 1996. Cliniical evidence of angiogenesis after arterioal gene transfer of phVEGF165 in patient with ischemic limb. Lancet 348:370–74.

Kibbe, M.R., Billiar, T.R., and Tzeng, E. 2000. Gene therapy for restenosis. Circ. Res. 86:829–33.

Kizana, E., Alexander, I.E. 2003. Cardiac gene therapy: Therapeutic potential and current progress. Curr. Gene Ther. 3:418–51.

Lafont, A., Durand, E., Vilde, F., et al. 1998. Thrombus generation after adenovirus-mediated gene transfer into atherosclerotic arteries. Hum. Gene Ther. 9:2795–2800.

Lai, N.C., Roth, D.M., Gao, M.H., et al. 2000. Intracoronary delivery of adenovirus encoding adenylyl cyclase VI increases left ventricular function and cAMP-generating capacity. Circulation 102:2396–2401.

Laitenen, M., Hartikainen, J., Hiltunen, M.O., et al. 2000. Catheter-mediated vascular endothelial growth factor gene transfer to human coronary arteries after angioplasty. Human Gene Ther. 11:263–70.

Losordo, D.W., Vale, P.R., Symes, J.F., et al. 1998. Gene therapy for myocardial angiogenesis: initial clinical results with direct myocardial injection of phVEGF165 as sole therapy for myocardial ischemia. Circulation 98:2800–04.

Mack, C.A., Patel, S.R., Schwarz, E.A., et al. 1998. Biologic bypass with the use of adenovirus-mediated gene transfer of the complementary deoxyribonucleic acid for vascular endothelial growth factor 121 improves myocardial perfusion and function in the ischemic porcine heart. J. Thorac. Cardiovasc. Surg. 115:168–76.

Mäkinen, K., Manninen, H., Hedman, M., et al. 2002. Increased vascularity detected by digital subtraction angiography after VEGF gene transfer to human lower limb artery. A randomized, placebo-controlled, double-blinded phase II study. Mol. Ther. 6:127–33.

Mann, M.J., Whittemore, A.D., Donaldson, M.C., et al. 1999. Ex vivo gene therapy of human vascular bypass grafts with E2F decoy: the PREVENT single-centre, randomized, controlled trial. Lancet 354:1493–8.

Meager, A. 2003. Quality and safety of gene medicines: a practical guide. Davis-Horwood International, Goldaming, Surrey, UK.

Morice, M.C., Serruys, P.W., Sousa, J.E., et al. 2000. A randomized comparison of a sirolimus-eluting stent with a standard stent for coronary revascularization. N. Engl. J. Med. 346:1773–80.

Morishita, R., Gibbons, G.H., Horiuchi, M., et al. 1995. A gene therapy strategy using a transcription factor decoy of the E2F binding site inhibits smooth muscle proliferation *in vivo*. Proc. Natl. Acad. Sci. USA 92:5855–59.

Rosengart, T.K., Lee, L.Y., Patel, S.R., et al. 1999. Six-month assessment of a phase I trial of angiogenic gene

therapy for the treatment of coronary artery disease using direct intramyocardial administration of an adenovirus vector expressing the $VEGF_{121}$ cDNA. Ann. Surg. 230:466–70.

Stewart et al. 2002. Late breaker session, American Heart Association, Chicago.

Symes, J.F., Losordo, D.W., Vale, P.R., et al. 1999. Gene therapy with vascular endothelial growth factor for inoperable coronary artery disease. Ann. Thorac. Surg. 68:830–36.

Tio, R.A., Tkebuchava, T., Scheuermann, T.H., et al. 1999. Intramyocardial gene therapy with naked DNA encoding vascular endothelial growth factor improves collateral flow to ischemic myocardium. Hum. Gene Ther. 10:2953–60

Vale, P.R., Losordo, D.W., Millikin, C.E., et al. 2000. Left ventricular electromechanical mapping to assess efficacy of $phVEGF_{165}$ gene transfer for therapeutic angiogenesis in chronic myocardial ischemia. Circulation 102:965–74.

C. Cancer

Although gene therapy technology offers tremendous promise for cancer therapy, so far the results in the clinic have been disappointing. Initially, most biotech start-up companies committed to gene transfer technologies were aiming to develop curative therapies for solid tumours. After 14 years of trials and tribulations very few of them are left, but even now gene therapy protocols targeting cancer make up over half of the 113 open gene therapy protocols in the United States.

In the main, five different gene transfer approaches have been used in cancer patients: (i) to enhance the immune response to tumour antigens, e.g. in melanoma patients, which a account for 54 % of immunotherapy trials. (ii) to transduce tumours *in vivo* with the tumour suppressor genes, e.g., p53. (iii) to transfer a suicide gene, e.g., herpes simplex virus thymidine kinase (HSV-tk) into tumour cells that activate prodrugs, e.g., ganciclovir, to cytotoxic products. (iv) to express drug resistance genes in bone marrow cells to protect them from augmented chemotherapy. (v) to use conditionally replication-competent viruses to infect and destroy tumour cells, but leave normal cells unharmed. Some of these approaches are discussed below.

Immunotherapy for cancer

Several attempts have been made to introduce tumour antigens *ex vivo* in dendritic cells (DC), before returning the DCs to the host, or to inoculate lethally irradiated tumour cells modified to secrete cytokines, e.g., IL-2 or GM-CSF, that promote the accumulation of DCs, or to combine these approaches in order to stimulate immune responses against tumours. Despite proof of concept for such 'tumour vaccines' in preclinical mouse models, most vaccinated patients exhibit only weak or undetectable responses and no clinical benefit. To induce rejection of cancer-associated antigen-bearing tumour cells, tumour reactive lymphocytes must be capable of reaching the tumour site, be able to exit the vascular system and be capable of destroying the tumour cells. However, since most human cancer antigens are normal, unmutated differentiation molecules or unmutated proteins found only in tumour or germ cells, the immune system is tolerized to these. In fact large numbers of circulating T-lymphocytes capable of recognizing cancer antigens can be present in cancer patients without any biological effect, suggesting those T-cells are not avid enough, are tolerized or anergic, and thus unable to interact with tumour cells.

It is possible however to use integrating vectors to genetically modify T-cells *ex vivo* and infuse these effector cells with enhanced tumour recognition. Several preclinical studies have emphasized the importance of prior host manipulation, e.g., by eliminating host lymphocytes including regulatory T-cells, to provide tumour-killing lymphocytes with an optimal environment. In one approach, T-cell recognition of tumours is achieved by expressing high-affinity chimeric transmembrane receptors comprising the external recognition structure of an antibody and the signalling domain of a T-cell receptor. Such T-cells recognize tumour antigens in an MHC-independent fashion like monoclonal antibodies, but still employ effectors mechanism of T-cells. Alternatively T-cell receptor genes can be inserted into T-cells of other patients with tumours expressing the same tumour antigens and MHC restricting allele, thus overcoming the need to isolate tumour reactive effector cells from each individual patient. Survival of the transferred cells can also be enhanced by the introduction of chimeric cytokine receptors that use cytokines such as GM-CSF. Alternatively, sequences which disrupt signalling normally serving to inhibit effector responses could be introduced into T-cells.

Much of the progress needed in this type of cancer gene therapy should come from immunological advances. Greater understanding of effector lymphocytes, as well as their tumour target cells, regulatory T cells, immune tolerance mechanisms, should assist the development of more effective protocols. Tumour cells themselves powerfully resist immune effector cells by down regulating target molecules on the surface, secreting immuno-suppressive factors, and preventing activation of anti-tumour precursor cells. Thus more needs to be done to elucidate mechanisms whereby tumour cells protect themselves from immune attack.

Tumour Suppressors

The introduction (by vectors) of tumour suppressor genes into tumour cells predisposes that their

expression will induce growth arrest or apoptosis. Several known tumour suppressor genes have been used such as APC1, RB2, INK4A, PTEN6, ARF10 and p53. It is assumed firstly however that the restoration of a single genetic defect will be effective in inhibiting tumour cells that have many additional defects. Secondly, it is assumed the expression of added tumour suppressor genes does not have any detrimental effect in normal cells. Furthermore, a major hurdle to this approach is that every cell in a tumour would need to be infected and transduced – an enormous technical obstacle, particularly for disseminated cancer. Clinical trials using this approach have proven safety, documented some rare tumour regressions, but without showing any significant clinical benefit.

Suicide Gene Transfer

Several investigators have used viral vectors to deliver 'suicide genes' to cancer cells. Suicide genes encode enzymes that metabolize a harmless prodrug into a potent cytotoxic metabolite that can diffuse to neighbouring tumour cells. Several enzyme/ prodrug combinations have been evaluated, but HSV-tk has been the most widely used and has progressed furthest in clinical trials. HSV-tk converts ganciclovir into its phosphorylated form, which blocks DNA syntheses when incorporated into DNA. Suicide gene therapy protocols have shown that while these agents are safe, they are not sufficiently effective for tumour cell eradication. A phase III clinical trial of glioma patients using retrovirus encoded HSV-tk showed no clinical benefit.

Replication Competent Viruses

Since *in vivo* applications of non-viral or replication-incompetent viral vectors were associated with very limited transduction efficiency, the use of cytolytic replication-competent recombinant viruses has been increasingly considered. In this approach, infected tumour cells are killed and release progeny viruses to spread from inoculation sites to neighbouring cells, which are then also killed. The success of this approach depends on the ability to engineer or select viruses that replicate specifically and efficiently in tumour cells, while normal cells are spared, and further spread through the tumour. The first engineered 'oncolytic virus' to enter clinical testing was a defective herpes simplex virus mutant (G207) that lacked a virulence gene ICP34.5 to improve safety, and the gene encoding ribonucleotide reductase to restrict replication to dividing cells only. In a phase I clinical trial for malignant glioma, its direct injection into tumours was found safe and well-tolerated, but without significant clinical benefit.

An attenuated adenovirus lacking the early region protein E1B 55K, which normally binds and inactivates cellular p53 to allow efficient viral replication, cannot, in theory, replicate in normal cells, but can in tumour cells with mutated inactive p53. However, the specificity of this mutant adenovirus for p53 deficient cells remains very controversial; there is some evidence of its replication in normal cells having wild type p53. Nevertheless, in a phase II trial in patients with recurrent head and neck cancer, its intratumoral administration combined with chemotherapy (5-fluorouracil and cisplatin) showed promising results. Other replication competent viruses, e.g., an adenovirus in which the prostate specific antigen (PSA) promoter drives E1A, are under clinical investigation, but none have delivered convincing patient benefit yet.

The Coxsackie/Adenovirus-receptor (CAR) is a cell surface adhesion protein expressed at high levels in liver, kidney, brain, heart, pancreas, colon, and prostate but not in lymphocytes, spleen, skeletal muscles and fibroblast. One innovative strategy is to retarget adenoviruses from CAR to tumour cells by engineering ligands into viral proteins that use tumour cell surface proteins as new receptors. Another is to increase binding of virus particles to integrins or to enhance CAR independent non-specific attachment. So far, trials of these modified adenoviruses are at initial stages and conclusions of their usefulness are yet to be drawn.

Safety Assessment

The problems and risks for cancer patients treated within gene therapy trials will differ from the ones patients with other diseases have encountered. For instance, the risk-benefit ratio is different for terminal cancer than that for less life-threatening diseases. None of the severe adverse events (SAEs), which made it into newspaper headlines, occurred in cancer patients. Since tumour vaccine strategies often use cells modified *ex vivo*, safety testing can be done before infusion.

Databases on clinical trials in human gene transfer

Over nine hundred (918) Clinical Gene therapy trials have been reported around the world; 85% of those have dealt with cancer. Policy on the public availability of data held by regulatory agencies varies widely from country to country. However, it is in the public interest to provide access to the comprehensive information concerning these trials. Increasingly, databases of clinical trials acting as resource and analytical tools for scientists, research participants, sponsors, institutional oversight committees, federal officials, and others with an interest in clinical gene transfer, are being compiled and made available. These include:

The USA NIH/FDA Genetic Modification Clinical Research Information System (GeMCRIS) (http://www4.od.nih.gov/oba/Rdna.htm) for all NIH funded trials,

The German Ministry of Education and Research database (http://zks.uni-freiburg.de/dereg.html),

The UK Gene Therapy Advisory Committee (GTAC), (http://www.advisorybodies.doh.gov.uk/genetics/gta/publications.htm),

The Belgian authorities (http://www.biosafety.be/GT/ regulatory/GTtrials.html) for trials done in these countries.

The Journal of Gene Medicine Clinical Trial site at http://www.wiley.co.uk/genmed/clinical also provides comprehensive data on gene therapy clinical trials, worldwide.

Selected references

Fillat C, Carrio M, Cascante A, Sangro B. Suicide gene therapy mediated by the Herpes Simplex virus thymidine kinase gene/Ganciclovir system: fifteen years of application. Curr Gene Ther. 2003; 3: 13–26.

Gottesman MM. Cancer gene therapy: an awkward adolescence. Cancer Gene Ther. 2003; 10: 501–8.

NabelGJ. Cancergenetherapy: present status and future directions. Ernst Schering Res Found Workshop. 2003; 43: 81–8.

Nevin N. & Spink, J. 'Gene Therapy Advisory Committee Long Term Monitoring of Patients Participating in Gene Therapy.' *Human Gene Therapy*, 2000, 11:1253–1255

Scanlon KJ. Cancer gene therapy: challenges and opportunities. Anticancer Res. 2004; 24: 501–4.

Appendix 2- *Prophylactic vectored vaccines and DNA vaccines*

Vectored vaccines

In theory, any micro-organism (viral, bacterial or protozoan) can be genetically engineered to express heterologous antigen(s) in a foreign host; these are known as vectored vaccines. In practice, most vectored vaccines are derived from relatively few well- characterized viruses such as adenoviruses, poxviruses (modified vaccinia Ankara [MVA], avianpox), adeno-associated virus, Semliki Forest virus, Venezuelan equine encephalitis, Sindbis virus, bluetongue virus, retroviruses, herpesviruses and yellow fever virus, or from bacteria such as salmonella, BCG and shigella (usually expressing viral antigens). Although many are replication incompetent, essentially they are analogous to live attenuated vaccines.

Several vectored vaccines are already in clinical trial usage, e.g. engineered modified vaccinia Ankara (MVA) poxvirus in HIV and malaria trials, canarypox (attenuated for humans by virtue of being an avian specific virus) in trials with genes expressing antigens from rabies, measles, cytomegalovirus, Japanese encephalitis (JE) or HIV. The 17D attenuated strain of yellow fever (YF) virus as a vector expressing the surface glycoprotein of Dengue virus, JE or West Nile virus (WNV) is also at the clinical trial stage. Unlike many vectors where the heterologous antigen is expressed alongside the vector's own antigens, the YF surface antigen is replaced by the antigen of the alternative (vaccine) virus. For 17D constructs this has the advantage of not raising neutralising antibodies to the basic vector, whereas for other systems, there may be a limitation due to pre-existing antibodies to the vector. The RNA replicons based on togaviruses such as Semliki Forest virus are also likely to enter trials soon and an adenovirus and an adeno-associated virus expressing HIV antigens are also being developed.

In the absence of clear guidance from regulatory authorities, extensive quality and pre-clinical control testing of vectored vaccines is being performed by the manufacturers: testing for genetic stability, the possibility of integration and the need for neurovirulence studies is being highlighted. Biodistribution studies are generally being performed and, at least for an adenovirus vector, integration studies also. However, in many cases, appropriate animal test systems are unavailable or difficult to use, e.g. administering a human dose to a small rodent. As with some live viral vaccines, the level of reversion, e.g., from replication incompetent- to replication competent-virus, should be assessed

Uniquely, a vectored vaccine is a completely novel virus and the risk to the environment will be an essential element in its control. EU GMO directives[i] state an environmental risk assessment must accompany any dossier application for a clinical trial or licence application. However, different EU member states have implemented these directives either as 'deliberate release into the environment' or 'contained use'. Since the latter is more favourable to the setting up of clinical trials, countries using 'contained use' procedures, e.g. the UK, are chosen by industrial developers more often for trials of vectored vaccines. Interestingly, shedding of pox viruses does not appear to be regarded as an issue since pox virus vectors are in high use in the veterinary field and there appears to be little concern regarding this.

DNA vaccines

Besides vectored and conventional vaccines, direct intramuscular injection of naked plasmid DNA encoding a foreign antigenic protein has been shown to generate an immune response to that antigen. This 'DNA vaccine' approach has several advantages over conventional vaccines; plasmid DNA can be rapidly manufactured and purified, is stable and non-infectious, does not replicate, and expresses *de novo* only the desired protein in its native conformation, thus generating functionally relevant antibody responses. Therefore, DNA vaccines not only provide safer alternatives to immunization with live attenuated viruses, but may also provide increased protective efficacy relative to inactivated or protein subunit vaccines.

Accumulated laboratory data have now shown that DNA vaccines evoke protective immunity against several infectious, mainly viral, diseases. The speed of their development has been such that clinical trials,

e.g., using DNA vaccines against HIV, HBV, influenza and malaria, and have already taken place. HIV DNA vaccine trials initially involved plasmids encoding an HIV viral protein(s), but specific antibody responses were poor and transient. Subsequently, T cell epitopes – small peptide regions that stimulate a specific T cell response – have been incorporated into HIV DNA vaccines. This approach has also been followed with malaria DNA vaccines and in trials, T cell responses are being obtained but not antibody responses.

The effectiveness DNA vaccines in the clinic has, to-date, generally been disappointing. Therefore, modifications to improve the uptake, e.g., microparticle bombardment ('gene gun'), electroporation, combination of DNA with various biopolymers to yield lipoplexes or polyplexes, and/or the immunogenicity, e.g., by optimisation of codon usage, use of molecular adjuvants (plasmids encoding immune stimulatory molecules such as cytokines), other adjuvants such as CpG motifs, and, significantly, the prime-boost approach (see below), of these novel vaccines are being investigated.

Nevertheless, clinical trials have not been in vain. They have clearly allayed concerns about the safety of DNA vaccines, especially with respect to tumourigenicity, immunopathology and induction of auto-immunity, and shown them to be well- tolerated in humans. There is evidence that chromosomal integration of plasmid DNA can occur, but at a level that is below that of naturally occurring spontaneous mutations. Also, in clinical trials, elicitation of anti-DNA antibodies or occurrence of serious adverse events has not been reported.

Regulatory guidance for DNA vaccines is adequate, first guidance documents from the WHO[ii] and the US FDA[iii] becoming available shortly after the onset of their development. In the EU, an all encompassing guideline on gene transfer products[iv], developed in the late 1990's, provides quality, non-clinical and clinical guidance for DNA vaccines.

The Prime/Boost Approach

Interestingly, the way forward for DNA vaccines could be to use them in combination with vectored vaccines – the 'prime-boost' approach. In comparing the efficacy of DNA vaccines with vectored vaccines in the laboratory, researchers discovered that the most effective course of vaccination was an initial dose (prime) with a DNA vaccine followed by a second dose (boost) with a vectored vaccine expressing the same antigen. The reciprocal approach, or the use of DNA alone or a viral vector alone, was less efficacious. This prime-boost approach has had some success in the clinic with HIV and malaria as the target organisms where, in both cases, the viral vector for the boost is an engineered pox virus vector (usually MVA, although canarypox has also been investigated). Both HIV and malaria studies of this nature have been pioneered at Oxford and the prime/boost approach is being followed by many researchers world-wide. A further novel finding has also recently emerged from the Oxford laboratories and that is that a prime/boost protocol in which the individual is primed with a pox vector and boosted with a distinct pox vector expressing the same antigen, is more efficacious than a DNA vaccine/pox vector prime/boost schedule. Quite clearly, this area of research still has far to go.

References

[i] Council Directive 90/219/EEC on the *contained use* of genetically modified micro-organisms, as amended by Directive 98/81/EC.

[3] Directive 2001/18/EC on the *deliberate release* into the environment of genetically modified organisms. Commission Decision 2002/623/EC establishes guidance notes supplementing Annex II of directive 2001/18/EC.

[ii] Guidelines for assuring the quality of DNA vaccines. WHO Technical Report Series 878, 1998, pp77–90. WHO, Geneva.

[iii] FDA Points to Consider on Plasmid DNA Vaccines for Preventive Infectious Disease Indications (1996).

[iv] CPMP Note for Guidance on the Quality, Preclinical and Clinical Aspects of Gene Transfer Medicinal Products, CPMP/BWP/3088/99.