From the Division of Hematology, Department of Medicine, and
Clinical Research Centre, Karolinska Institutet at
Huddinge University Hospital, Stockholm, Sweden

Vector development for suicide gene therapy

Alar Aints

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To Liis, Karl Mihkel
and Hermine

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ABSTRACT

Gene therapy is used to treat conditions that arise from errors in the genetic makeup of cells – either congenital diseases resulting from a deletion or mutation in a gene or malignant diseases where genetic regulation mechanisms have been deranged.

Suicide gene therapy is one of several gene therapeutic approaches to treat cancer. A suicide gene is a gene encoding a protein, frequently an enzyme, that in itself is non-toxic to the genetically modified cell. However, when the cell is exposed to a specific non-toxic prodrug, this is selectively converted by the gene product into toxic metabolites that kill the cell.

The gene transfer technologies available today have limited effectiveness – only a fraction of target cells can be genetically modified. This can be compensated by several processes taking place after transgene expression and enzymatic conversion of the prodrug. *In vivo*, and also in cell culture, gap junction mediated intercellular communication (GJIC) enables the spread the converted metabolites. However, many tumours lacking connexin expression are not susceptible to this form of treatment.

To bypass the requirement for gap junctions, one can link the HSV-tk gene to the gene of another herpes virus protein, VP22. The VP22 protein has been shown to pass freely between cells. We show the ability of VP22-GFP fusion protein to spread in cell culture and translocate into the nucleus of the recipient cells (Paper I). Also, VP22 fusion to TK (VP22-TK) is transferred to the surrounding cells in cell culture and *in vivo* in a mouse tumour model, in the absence of gap junction formation in a connexin negative tumour cell line and sensitises the tumours to GCV. However, relatively high proportion (50%) of VP22-TK expressing cells is necessary to exert a full effect (Paper II). Therefore, we have analysed the VP22 protein in detail. Mapping of functional domains revealed several independent functional domains and localisation signals in the VP22 polypeptide. These constructs can have a potential use in targeting defined functional peptides to specific subcellular locations (Paper III).

Suicide gene technology can provide a safety switch for cytotoxic effector cells, to treat graft versus host disease (GvHD). In this application, it would be ideal to achieve 100% effectiveness of modification of the target cells. Different selection marker genes for selection have been utilised, and these fall into two categories: metabolic and physical selection markers. As there is requirement for a resistance marker better suited for selection of human cells, we have characterised the ouabain resistance gene (OuaR).

OuaR belongs to the Na^+,K^+-ATPase gene family - a housekeeping enzyme present in all mammalian cells. It maintains cellular homeostasis of Na^+ and K^+. Ouabain belongs to cardiac glucoside group of drugs. It inactivates the naturally occurring non-resistant Na^+,K^+-ATPase which results in very rapid cell death. OuaR is a PCR-generated point mutant L799C that is completely resistant to inhibition by ouabain and can be used for background free selection in very short time – 12-36 hours, compared to 10-15 days for classical antibiotic selection. We show the versatility of OuaR gene transfer by transient transfection and stable retrovirus-mediated integration in several cultured cell lines as well as primary human donor T-lymphocytes (Paper IV).

We have prepared suicide gene therapy vectors containing HSV-tk and OuaR selection marker fusion gene in extensively optimised retroviral backbone SF91. The OuaR selection marker allows the transduced cells to be selected chemically in 36 hours, reducing the necessary time for *in vitro* culture to a week. The transduced T-cells display high sensitivity to gancyclovir. The rapid gene transfer and selection process prevents culture-related changes in T-cell function and helps to establish protocols to gain control over cytotoxic cells by suicide gene transfer (Paper V).

In conclusion, we have characterised vectors for suicide gene therapy with the potential to achieve 100% efficiency of genetic modification of the target cells. VP22 protein vectors enable the spread of fusion protein to all surrounding cells after gene expression in the gene-modified cells. Viral or plasmid vectors containing the OuaR selection marker allow the gene-modified cells to be selected immediately after gene expression, reducing the time for selection and time-dependent *in vitro* cell culture-related side-effects to minimum.

LIST OF ORIGINAL PUBLICATIONS

I **Aints A**, Dilber MS, Smith CIE. Intercellular spread of GFP-VP22. J Gene Med 1999, 1:275-279.

II Dilber MS, Phelan A, **Aints A**, Mohamed AJ, Elliott G, Smith CIE and O'Hare P. Intercellular delivery of thymidine kinase pro-drug activating enzyme by the herpes simplex virus protein VP22. Gene Ther 1999, 6: 12-21

III **Aints A**, Güven H, Gahrton G, Smith CIE and Dilber MS. Mapping of herpes simplex virus-1 VP22 functional domains for inter-and subcellular protein targeting. Gene Therapy 2001, 8: 1051-1056.

IV **Aints A**, Belusa R, Andersson R, Güven H, Dilber MS. Enhanced ouabain resistance gene as an eukaryotic selection marker. Human Gene Therapy. In Press.

V **Aints A**, Unger C, Rabbani H, Dilber MS. Rapid suicide gene transfer and selection of primary human T-cells. Manuscript.

LIST OF ABBREVIATIONS

AAV – adeno-associated virus
ADA – adenosine deaminase [defficiency]
AICD – activation induced cell death
ALL – acute lymphoid leukaemia
AML – acute myeloid leukaemia
APC – antigen presenting cell
ATPase – adenosine triphosphatase
AV – adenovirus
BMT – bone marrow transplantation
CD – cluster of differentiation
CD – cytosine deaminase
cDNA – complementary DNA
CEA – carcinoembryonic antigen
CFU – colony-forming unit
CLL – chronic lymphoid leukaemia
CML – chronic myeloid leukaemia
CMV – cytomegalovirus
CsA - cyclosporin A
CTE – constitutive transport element
Cx – connexin
DC – dendritic cell
DHFR – dihydrofolate reductase
DLI – donor lymphocyte infusion
DMEM – Dulbecco's modified Eagle's medium
EBV – Epstein-Barr virus
EF1α – elongation factor 1 α subunit
FACS – fluorescence activated cell sorting
FCS – foetal calf serum
FMDV – foot-and mouth disease virus
GALV – gibbon ape leukaemia virus
GCV – gancyclovir
GFP – green fluorescent protein
GJIC – gap junction intercellular communication
GM-CSF – granulocyte-macrophage colony stimulating factor
GvHD – Graft-versus host disease
HIV – human immunodeficiency virus
HPRT – hypoxanthine phosphoribosyl transferase
HSCT – hematopoietic stem cell transplantation
HSV – herpes simplex virus
i.m. – intramuscular
i.p. – intraperitoneal
i.v. – intravenous
i/e – immediate/early
IFN – interferon
IL – interleukin
IRES – internal ribosome entry site
ITR – inverted terminal repeat
IU – infectious unit
kb – kilobasepairs
kD – kilodaltons
LCMV-G – lymphocytic chorio-meningitis virus glycoprotein
LTC-IC – long-term culture initiating cell
LTR – long terminal repeat
MACS – magnetic cell sorting
MDR – multidrug resistance
MESV – Murine embryonic stem cell virus
MHC – major histocompatibility complex
MMLV – Moloney murine leukaemia virus
mRNA – messenger RNA
MTX – methotrexate
NeoR – neomycin resistance gene
NGFR – nerve growth factor receptor
NK – natural killer
NLS – nuclear localisation signal
ORF – open reading frame
OuaR – ouabain resistance gene
pA – polyadenylation signal
PBL – peripheral blood lymphocytes
PBS – phosphate buffered saline
PBS – primer binding site
PBSC – peripheral blood stem cells
PEG – polyethylene glycol
PEI – polyethylene imine
PFU – plaque-forming unit
R – receptor
rh – recombinant human [protein]
RPMI – Roswell Park Memorial Institute
s.c. – subcutaneous
SA – splice acceptor
SCID-X1 – severe combined immunodeficiency, X-linked, type 1
SD – splice donor
SFFV – Spleen focus-forming virus
SFV – Semliki Forest Virus
SIN – self-inactivating
SIN – Sindbis virus
SS – splice signal
SV40 – simian virus 40
TCD – T-cell depletion
TCR – T-cell receptor
TfR – transferrin receptor
TK – thymidine kinase
TNF – tumour necrosis factor
UTR – untranslated region
VEE – Venezuelan equine encephalitis virus
VP22 – viral protein 22
WPRE – woodchuck hepatitis virus RNA stability element
VSV-G – vesicular stomatitis virus glycoprotein
wt – wild type
Ψ – packaging signal

INTRODUCTION

Gene therapy is used to treat conditions that arise from errors in the genetic makeup of cells – either congenital diseases resulting from a deletion or mutation in a gene or malignant diseases where genetic regulation mechanisms have been deranged. In some malignancies, new approaches to tumour therapy, such as gene-transfer based cytotoxic delivery systems, may provide an alternative or a complement as there is still a significant fraction of patients that will not benefit from the therapy available today. Gene therapy can be broadly divided into therapy for genetic disease, when the objective of therapy is to restore a missing function; and gene therapy for cancer, where the aim is to kill the malignant cells, and spare the normal ones.

The success of gene therapy is largely dependent on the success of gene transfer. Several types of vectors for transfer of genetic material exist.

GENE TRANSFER TECHNOLOGIES

Genetic information is encoded in large negatively charged polymer molecules, DNA and RNA. The uptake of pure DNA into mammalian cells is very inefficient, although intramuscular injection of circular plasmid DNA is sufficient for DNA immunisation. For more efficient uptake, electrical pulses can be used (electroporation) and this results in efficient uptake of foreign DNA in the cells. The pulse parameters have to be optimised for each cell type, and this technique is difficult to apply *in vivo*. For more efficient uptake, DNA or RNA can be complexed with positively charged chemicals, which protect the DNA from degradation and facilitate the transport across cellular membranes.

Chemical methods

The first chemical reagent for gene transfer was calcium phosphate. DNA can be co-precipitated at a sharply defined pH (7.04) When applied to cells, it is taken up, released from endosomes and transferred to the nucleus. Several more efficient chemical transfection reagents have been developed since then, such as cationic polymers (polylysine, polyethylene imine) (Boussif *et al.*, 1995) and cationic lipids.

During transfection, the incoming DNA must cross several barriers before gene expression can occur. **First**, the cell membrane. In order to enter the cell, the transfection complex is bound to the cell membrane, after which it is endocytosed. This offers possibilities for targeting the gene transfer to certain cell types by receptor-ligand interactions. Transferrin, galactose (Zanta *et al.*, 1997) and specific antibodies have been used successfully. **Secondly**, escape from endosome. Different strategies can be used – endosome buffering and following osmotic rupture in PEI, or virally derived peptides for pH-dependent membrane fusion. **Thirdly**, import into nucleus can be enhanced by linking DNA to specific nuclear localisation peptides (Branden *et al.*, 1999). Despite several advanced methods, chemical DNA transfer remains inefficient in comparison with viral gene transfer. Only a few established cell lines (293, Cos, 3T3, HeLa) can be transfected at 50-80% efficiency under optimal conditions. For other adherent cell lines, the efficiency remains around 5-15%, and only 1-5% for non-adherent cell lines. Primary cells are generally resistant to chemical methods of gene transfer.

Viral gene transfer vectors

Viral vectors, on the other hand, provide very high efficiency for gene transfer in culture, and are also highly efficient *in vivo*. Several different virus types have been used for constructing gene therapy vectors. As viruses utilise several different methods of encoding genetic information, and transfer, different vectors exhibit various advantages and disadvantages for particular purposes. Table 1 summarises some of the practical points of more common gene transfer vectors.

Table 1. Viral vectors.

Vector group	Genome	Capacity	Persistence	Titre $\log_{10}$ IU /ml	Immuno-genicity
AAV	ssDNA(Linear)	4-5 kb	Integrated tandem	11	low
AdV	dsDNA(Linear)	8 kb (E3) 28-32 kb (gutless)	Transient episomal	13	high
HSV-1	dsDNA(Linear)	30 kb 15 kb (amplicon)	Episomal	8 (conc.)	low
SV40	dsDNA(Circular)	4.7 kb	Episomal	10	low
Alphavirus	+RNA	4-5 kb	No	7	high
Retrovirus	+RNA	8 kb	Integrated	6-7	low
Lentivirus	+RNA	8 kb	Integrated	10 (conc.)	low

Adenovirus vectors

The genome of adenovirus consists of double stranded DNA of ~36 kilobases (kb). Adenovirus can infect a wide range of cell types, including non-dividing cells. Recombinant adenoviral vectors are derived from E1 deleted virus. Inactivation of other early genes further limits the viral replication and gene expression. Recently, other regions, like E4 have been deleted as well. The transgene is inserted in place of E3 region, which is dispensable for viral life cycle. Moreover, 'gutless' vectors containing no viral genes, have been developed. These vectors contain only viral terminal repeats and packaging signals, and thus exhibit significantly reduced immune responses. The vectors can only be grown in the presence of a helper virus, which is a packaging defective first generation adenovirus lacking the E1 region. Up to 28–32 kb can be inserted into gutless vectors. The helper virus and vector can be further purified by physical means. This process is currently labor intensive and difficult to scale up. Contaminating helper viruses approach levels of less than 0.1% (Kay *et al.*, 2001).

Current Adenovirus vectors are primarily derived from serotypes 2 and 5 - the most common serotypes to which most adults have been exposed. However, there are over 50 different human adenoviral serotypes with varied natural tropism. There are now efforts to exploit these other serotypes or even non-human adenoviruses, to avoid potential problems related to pre-existing immunity that may preclude or reduce the

efficacy of vector administration. Moreover, if and when re-administration of a vector may be required, secondary vector delivery using a different serotype capsid can be used. Also, retargeting of adenoviral particles is feasible using polymers and engineered ligands (Silman and Fooks, 2000).

Adeno-associated virus vectors

Adeno-associated virus belongs to the parvovirus family. It is considered nonpathogenic and requires adenovirus or herpesvirus for replication. The viral genome consists of two genes, each producing multiple polypeptides: rep, required for viral genome replication; and cap, encoding structural proteins. These two genes are flanked by viral ITRs that are 145 nucleotides in length. Each particle contains a single plus- or minus-strand genome. The packaging capacity of AAV is about 5.0 kb, which is a major limitation of this vector system (Kay *et al.*, 2001). The wild-type virus in the presence of rep has a propensity to integrate into a specific region of human chromosome 19. This property is lost, however in the absence of rep. The AAVS1 integration site on Chr-19 has been linked recently to the TNNT1 gene, encoding troponin I isoforms normally expressed in slow skeletal muscle. Investigations in latently infected cell lines have demonstrated integrated wt AAV sequences disrupting the TNNT1 gene. Whether this event would represent a risk in (multinucleated) muscle cells or elsewhere is unclear. (Carter and Samulski, 2000; Monahan and Samulski, 2000)

80% of all cDNAs fall within the 3–6-kb size range. rAAV delivery of genes nearing or exceeding 5 kb in size (including some that are the objects of intense investigation, e.g. dystrophin, factor VIII and the cystic fibrosis transmembrane conductance regulator (CFTR)) has been disappointing. Heterodimerization of separate rAAV vectors may provide a solution. It takes advantage of a unique property of wtAAV biology, namely, expression from latent AAV proviral DNA existing in head-to-tail concatemers. The 5' and 3' segments of a split expression construct will be rejoined through paired rAAV inverted terminal repeats. One of the vectors contains a splice donor, the other a splice acceptor signal. The ITRs are removed with intronic sequences during mRNA processing, resulting in a full-length transcript up to 10 kb. This approach may effectively double the cloning capacity for AAV vectors. (Carter and Samulski, 2000; Monahan and Samulski, 2000).

Herpesvirus vectors

Herpes simplex virus (HSV) is an enveloped double-stranded DNA virus. It is a complex virus with a 150 kb genome encoding ~80 genes. HSV is the vector with the largest loading capacity, about ~30 kb of foreign genes. Thus, HSV-based vectors can carry large DNA molecules like genes containing native promoters and locus control regions. HSV-1 is able to infect a wide range of cells. In the central nervous system HSV proceeds to latent infection. In this state, most of the viral genes are transcriptionally silent. This feature makes HSV a promising vector for gene transfer to neurons (Janson *et al.*, 2001) (Carlezon *et al.*, 2000). Furthermore, the HSV genome remains episomal after infection, so eliminating the possibility of opportunistic malignant insertional mutagenesis.

Amplicon plasmids are based on defective interfering virus genomes that arise on high passage of virus stocks. They are generally approximately 15 kb in length and minimally possess a viral origin of replication and packaging sequences. The standard amplicon system requires the functions of helper HSV for particle production and packaging of genome length concatemerized vector DNA. Amplicon vector production

has been improved through use of helper virus genome plasmids deleted for packaging signals; the helper genomes are propagated in bacteria as bacterial artificial chromosomes (Saeki *et al.*, 1998; Sena-Esteves *et al.*, 2000; Saeki *et al.*, 2001). However, amplicons yield 2-3 logs lower tires compared to genomic vectors (Kay *et al.*, 2001). Herpesvirus vectors are promising in gene transfer to nervous system.

Alphavirus vectors

Alphaviruses have the advantage of being exclusively RNA-based viruses and replicating entirely in the cell cytoplasm. They have several features that make them attractive as gene delivery platforms, particularly for suicide therapy and immunisation purposes (Gardner *et al.*, 2000; Polo and Bergmann, 2001; Vajdy *et al.*, 2001; Colmenero *et al.*, 2002; Lundstrom, 2002). Vectors are derived from Sindbis virus (SIN), Semliki Forest virus (SFV), and Venezuelan equine encephalitis virus (VEE). Alphavirus vectors, termed "replicons", retain the non-structural protein genes encoding the viral replicase, that in turn programme high level cytoplasmic amplification of the vector RNA cloned under the viral subgenomic promoter (Polo *et al.*, 2000). Recently, strains of Sindbis virus, capable of persistent infection in cell culture, were identified (Perri *et al.*, 2000). This has facilitated creation of stable packaging cell lines capable of producing Sindbis virus, as well as Semliki forest virus vector particles at high titres (Polo *et al.*, 1999).

SV40 vectors

The ability of SV40 to infect nondividing cells makes it an attractive candidate for gene delivery to quiescent cells, such as hepatocytes. By replacing the Tag gene with a target transgene, the vector becomes replication defective, non-transforming and non-immunogenic. This rSV40 system can accommodate up to 4.7 kb of exogenous DNA. rSV40 has been shown to transfer genes effectively into bone marrow progenitor cells, peripheral blood lymphocytes, and normal hepatocytes *in vivo* when infused into the portal vein. Replication competent virus was not detected. Also, SV40 can be considered non-pathogenic in humans, as was indicated by the long-term follow-up (40 years) of individuals who received early batches of Salk polio vaccine that was inadvertently contaminated with SV40 virus (Sauter *et al.*, 2000; Strayer, 2000).

Retrovirus vectors

Retroviruses are highly efficient in delivering genetic material, they integrate the transgene permanently into the target cell genome and elicit no vector-associated cellular immune response.

Most frequently used retroviral vectors are derived from Moloney Murine Leukaemia Virus (MMLV). This murine retrovirus is capable of integrating its transgene into human DNA and expressing the protein of interest from the viral LTR promoter or an internal promoter. One of the first genes to be transferred by retrovirus vectors were hypoxanthine phosphoribosyltransferase (HPRT), (Miller *et al.*, 1983), rat growth hormone gene (Miller *et al.*, 1984) and dihydrofolate reductase (Miller *et al.*, 1985). Components for retroviral vector production are listed in table 2.

Table. 2 Retroviral vector components

PACKAGING CELLS	CIS-ACTING ELEMENTS	ENVELOPES
Phoenix (293)	Promoter	Amphotropic
BOSC (293)	5'UTR	Ecotropic
PG13 (3T3)	Packaging sequence	10A1
PA317 (3T3)	SS	GALV
FLY (HT1080)	CTE	RD114
TE-FLY (TE671)		
APEX (ECV 304/T24)	IRES	VSV-G
	WPRE	LCMV-G

Packaging cells

Production of recombinant retroviral particles in a producer cell line (figure 1) can be done either transiently, by co-transfection of gag-pol, env and vector plasmids, or from a stable packaging cell line, harbouring these three plasmids stably integrated into chromosomes. For transient transfection, the 293 cell line is used most frequently, since a very high efficiency of transfection, approaching 60-70%, and high level of expression, can be achieved with this cell line. When constructing a stable cell-line, packaging constructs need to be on separate plasmids, in order to minimise risk for replication competent virus generation (Miller and Buttimore, 1986). Several stable cell lines, facilitating vector production, have been constructed, based on 293 cell line, containing either gag-pol (Phoenix GP), or gag-pol and env (Phoenix Ampho, Phoenix Eco) genes in separate transcription units (Kinsella and Nolan, 1996). Highly efficient transient production of retrovirus can be achieved by incorporating the EBV replication cassette on the vector plasmid, outside the retroviral LTRs (Kinsella and Nolan, 1996; Yang *et al.*, 1999). By selection, these cell lines can be converted to stable producer cell lines.

The other packaging cells used to generate retroviral particles are mainly based on mouse 3T3 fibroblasts (PA317 and PG13 (Kotani *et al.*, 1994)), and human fibrosarcoma cell lines HT1080 (FLY, (Cosset *et al.*, 1995)) and TE671 (TE-FLY) (Duisit *et al.*, 1999). Human cells have the advantage of not containing endogenous retroviruses, as the murine cells do, thus there is less risk of generating a replication-competent virus.

A packaging cell line called APEX was designed to produce vectors for HSC transduction. ECV 304/T24, the parental cell line of APEX, support the growth of human CD34(+) progenitor cells as primary human bone marrow stroma. The APEX-produced vectors reached 50% efficiency in transduction of primitive CD34+ long-term-culture initiating cells (Dando *et al.*, 2001).

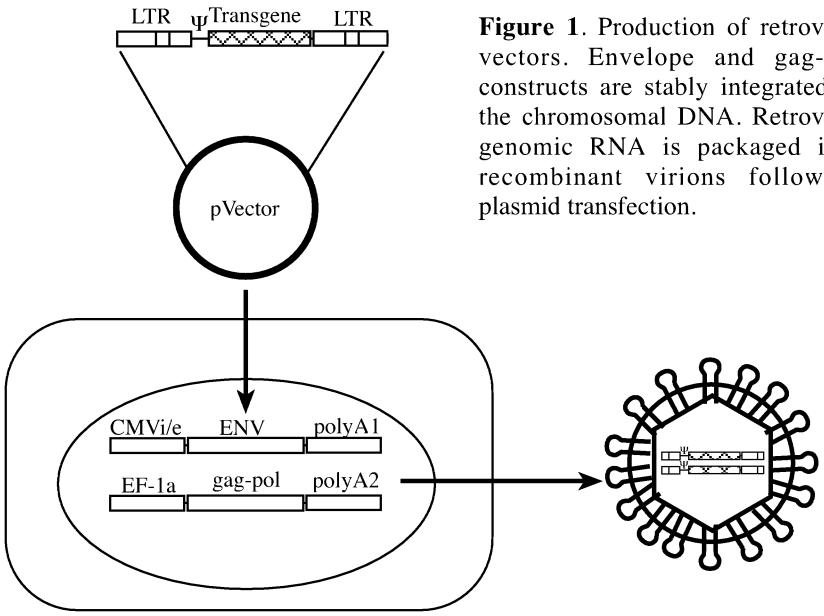


Figure 1. Production of retroviral vectors. Envelope and gag-pol constructs are stably integrated in the chromosomal DNA. Retroviral genomic RNA is packaged into recombinant virions following plasmid transfection.

Envelopes

Seven distinct receptor groups have been identified on human cells for C-type and D-type retroviruses. All the D-type simian retroviruses (SRV-1-5, SMRV, PO-1-Lu) share a common receptor which is also utilized by the baboon and cat endogenous C-type viruses (BaEV, RD114). Gibbon Ape leukaemia virus (GALV) and feline leukemia virus subgroup B (FeLV-B) belong to the second group. Human T-cell leukemia viruses types 1 and 2 (HTLV-1, HTLV-2) share a common receptor with related chimpanzee and simian viruses (STLV). Amphotropic and xenotropic murine leukemia viruses (MLV-A, MLV-X), bovine leukemia virus (BLV), and FeLV-C infect human cells via unique cell surface receptors (Sommerfelt and Weiss, 1990).

The C-type viruses simian sarcoma associated virus, feline leukemia virus subgroup B, and the feline endogenous virus RD114 are able to form pseudotypes with the Mo-MLV genome. However, human T cell leukemia virus-1 (HTLV-1) and the D-type viruses Mason-Pfizer monkey virus and simian retrovirus-1 fail to rescue the Mo-MLV vector (Takeuchi *et al.*, 1992).

The original MMLV envelopes of different tropism are continued to be used. The ecotropic envelope can infect only murine cells, the amphotropic envelope can infect both murine and other mammalian cells, including human. Several other envelopes have been characterised, which can be used for vector generation. They provide altered tropism, broader range or higher resistance to inactivation.

MuLV-10A1 envelope is highly efficient in transducing human cells. This correlates with a longer half-life of this pseudotype in comparison with A-MuLV and, as shown by interference analysis with the human T cell line HUT78, by the utilization of both the A-MuLV receptor (Pit2) and the GaLV receptor (Pit1) for cell entry (Uckert *et al.*, 2000).

Gibbon Ape Leukaemia Virus (GALV) envelope glycoprotein is more efficient for transduction of human T-cells and hematopoetic stem cells than

amphotropic envelope since the expression of Pit-1 is higher on the stem cells (von Kalle *et al.*, 1994).

Feline endogenous virus RD114 envelope is resistant to human serum complement (Cosset *et al.*, 1995; Porter *et al.*, 1996). Human repopulating cells from umbilical cord blood capable of establishing hematopoiesis in immunodeficient mice were efficiently transduced by RD114-pseudotyped particles, whereas amphotropic particles were ineffective at introducing the proviral genome. After only a single exposure of CD34(+) cord blood cells to RD114-pseudotyped particles, all engrafted nonobese diabetic/severe combined immunodeficiency mice (15 of 15) contained genetically modified human bone marrow cells. Human cells that were positive for enhanced green fluorescent protein represented as much as 90% of the graft (Kelly *et al.*, 2000; Kelly *et al.*, 2001).

Vesicular Stomatitis Virus glycoprotein (VSV-G) can pseudotype retroviral particles, and has a broad range. It is very efficient in transducing cultured cell lines. However, on primary human T-cells, the VSV-G virions require 10- to 100-fold higher concentrations of infectious particles to achieve levels of gene transfer comparable to GaLV-virions (Gallardo *et al.*, 1997). The VSV-G receptor is a cell membrane glycolipid, and the vector is internalised via endocytic pathway, in contrast to retroviral envelope proteins, which mediate cell membrane fusion. Continuous expression of VSV-G is toxic to the expressing cells, therefore an inducible promoter has to be used in the packaging cell line (Ory *et al.*, 1996).

Lymphocytic Chorio-Meningitis Virus (LCMV) is a murine arenavirus with broad cellular tropism. LCMV-G-pseudotyped oncoretroviral and lentiviral vectors can be produced in vector titers similar to those with A-MLV env or VSV-G. In contrast to A-MLV env particles, LCMV-G pseudotypes can be efficiently concentrated by ultracentrifugation without loss of vector titer. LCMV vectors have a broad tropism, the receptor for LCMV-G is alpha-dystroglycane (Beyer *et al.*, 2002).

Cis-acting elements

The cis-acting elements are specific sequence elements that determine the interactions of DNA and RNA with the cellular machinery.

Promoter

Retroviral promoter is located in the U3 region of the LTR. Different retroviral promoters have different activity in different cell types. Moloney murine leukemia (MMLV) LTR is highly active in lymphoid cells (Speck *et al.*, 1990). Spleen focus-forming virus (SFFV) LTR activity is highest in myelo-erythroid progenitor cells (Baum *et al.*, 1995; Eckert *et al.*, 1996).

5'Untranslated region (5'UTR)

The sequences of the primer-binding site (PBS) for $tRNA^{Pro}$ are found in both Moloney and FMCF-related viruses. It can inhibit retroviral transcription in embryonic stem cells (Petersen *et al.*, 1991; Kempler *et al.*, 1993) and in primitive hematopoietic cells (Baum *et al.*, 1995). In contrast, the murine embryonic stem cell virus (MESV) (PBS for $tRNA^{Gln}$) confers stable expression in these cell types (Grez *et al.*, 1990).
FMCF/MESV hybrid vectors (FMEVs) are generated by combining the SFFVp U3 with the PBS from MESV (Baum *et al.*, 1995). They provide high expression of transduced genes in primitive hematopoietic cells.

In MoMLV-based vectors the packaging sequence extends into gag coding region. To avoid expression of the gag protein, the initiating ATG has been mutated. However, several additional initiating codons present in the packaging sequence may initiate translation at low levels (Hildinger *et al.*, 1999).

Packaging sequence

The packaging efficiency of a retrovirus construct depends primarily on its size and the efficiency of the packaging sequence located in the 5'UTR. However, in the first retroviral constructs, the packaging sequence extends into the gag coding region, thus increasing the total size of the construct. Additional splice sites in the pol coding region have been added to enhance packaging and expression, as in MFG type of vectors (Podrazik *et al.*, 1992; Jaffee *et al.*, 1993; Krall *et al.*, 1996). A splice donor/splice acceptor pair in the 5' UTR is important for both efficient packaging of the genomic transcript and high level of expression, preventing the silencing of intronless transcripts. Generally, retroviruses can not transfer introns. However, splice signals with low processing efficiency are not removed (or, if they are, the RNA is not packaged)

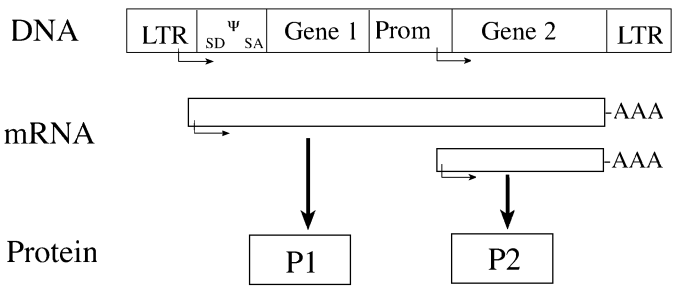
Additional cis-acting elements influence the RNA processing and stability, and therefore, the level of gene expression. The cis-acting elements from diverse viruses include **WPRE** (woodchuck hepatitis virus post-transcriptional RNA stability element) and **CTE** (constitutive transport element) from D-type retroviruses.

Splicing can improve expression considerably. Interactions between the cis-acting elements can be additive. WPRE, as well as CTE, improve the effect or compensate for the absence of a splice site, but WPRE can not compensate for the absence of CTE for genes that require this type of element. Therefore, the effects are also gene specific (Schambach *et al.*, 2000).

Principles of multigenic retroviral vector construction

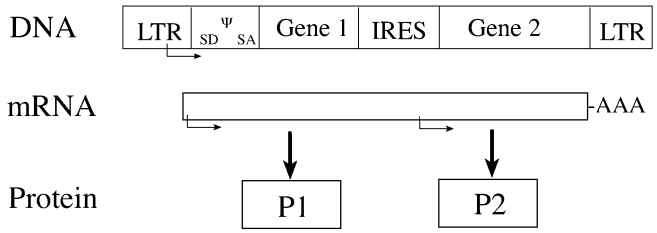
For suicide gene therapy it is generally necessary to express the suicide gene together with the selection marker. In order to ensure concomitant expression of the two reading frames from a single gene transfer construct, several transcriptional and translational control mechanisms can be utilised.

Internal promoters direct synthesis of a second mRNA, which directs the synthesis of the second ORF. The favoured promoters are SV40 , CMVi/e, and EF-1a promoters. The promoters are regulated independently. Usually, concordance of expression is around 70%.

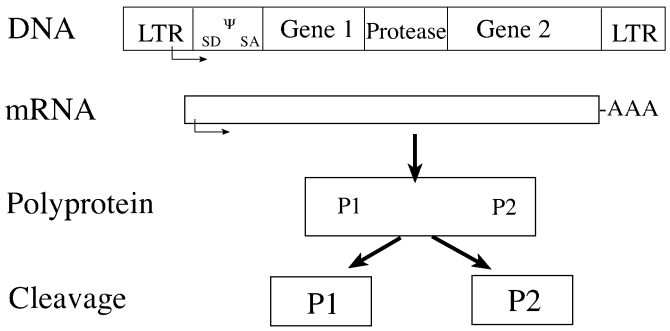


Internal Ribosome Entry Site (IRES) directs independent translation of the second reading frame from a single mRNA. IRES sequences have been isolated from

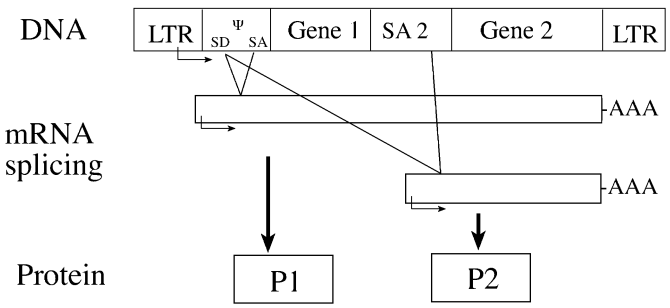
picornavirus (encephalomyocarditis virus (EMCV)) and poliovirus, but also from human genes, e.g. NFkB repressing factor (NRF) (Oumard *et al*., 2000).



Autocatalytic peptide cleavage site from foot and mouth disease virus (FMDV) will self-cleave post-translationally and release two polypeptides after translation of a single ORF (Klump *et al*., 2001).



Splice acceptor (SA) site with an intermediate affinity between the ORFs will enable the expression of two mRNA species and cap-dependent translation of the both reading frames. If there are no stop codons present in the SA and the two ORFs are cloned in-frame, a fusion peptide and the second partner will be expressed. If a stop codon is present in the SA, two independent peptides are expressed.



Lentivirus vectors

Lentiviruses belong to the retrovirus family. The best-known lentivirus is the human immunodeficiency virus (HIV-1). Lentiviruses can infect both dividing and non-dividing cells (Naldini *et al.*, 1996), which makes lentiviral vectors a favoured tool for gene therapy. Human hematopoietic stem cells (HSC) are virtually all in G0/G1. The HIV1- based vector, but not MuLV vector supernatants, can transduce freshly isolated HSC G0/G1 from mobilised peripheral blood. (Uchida *et al.*, 1998; Miyoshi *et al.*, 1999). Quiescent, G0/G1 HSC can be transduced, because lentiviruses (such as HIV type 1, HIV) encode proteins and cis-acting sequence elements that permit transport of the viral genome into the nucleus of nondividing cells (Follenzi *et al.*, 2000). The nuclear transport of preintegration complex is guided by nuclear localisation signals present in the viral matrix. Efficient transduction into various human cell types including human CD34+ cells has been demonstrated by using an HIV-1-based vector gene delivery system. Lentiviral vectors are also efficient delivery vectors *in vivo* for muscle, liver and neuronal cells (Zufferey *et al.*, 1997; Zufferey *et al.*, 1998; Naldini, 1999; Follenzi *et al.*, 2002). Up to 7.5 kb genes of interest can be inserted into the lentiviral vectors. HIV-1 has gag, pol, and env genes, but it also carries genes for six accessory proteins called tat, rev, vpr, vpu, nef and vif, which have been deleted in vector constructs.

Production of the lentiviral vectors proceeds by transient transfection of 293 cell line by four plasmids, containing the transfer construct, lentiviral gag-pol and rev, as well as VSV-G envelope genes. Recently, also a stable packaging cell line has been described, containing the plasmids under tetracycline-regulatable promoter. 293G cells express the chimeric Tet(R)/VP16 trans-activator and contain a tetracycline-regulated vesicular stomatitis virus protein G (VSV-G) envelope gene. After introduction of the transfer vector by serial infection, vector could be collected for several days before the cytopathic effect set in (Farson *et al.*, 2001).

Hybrid vector systems

As each vector system has its disadvantages, several attempts have been made to create hybrid vectors, utilising components from different viral replication strategies. For packaging of retroviral particles capable of incorporating elements for efficient RNA processing like introns and polyadenylation signals, a hybrid between Semliki Forest Virus and Moloney Murine Leukaemia Virus have been created. Retroviral structural proteins and the gene transfer construct containing an intron and a polyA signal were cloned on separate constructs under the SFV subgenomic promoter. After *in vitro* RNA transcription and electroporation into BHK cells, retroviral particles were produced, which contained introns and internal polyA signals (Li and Garoff, 1996, 1998).

The same strategy as been used in adenovirus (Reynolds *et al.*, 1999) and Vaccinia virus (Zanta *et al.*, 1997) based hybrid production system, which gives rise to retroviral particles. Since adenovirus can be produced at much higher titres than retrovirus, but cannot integrate, the retroviral vector produced *in situ* can provide stable gene expression in the second phase of transduction.

Also, hybrid production systems between baculovirus and AAV (Palombo *et al.*, 1998), and herpesvirus and AAV (Fraefel *et al.*, 1997; Johnston *et al.*, 1997) have been described.

Replicating oncolytic viruses

Since gene transfer efficiency using the viral vectors never reaches 100% efficiency *in vivo*, and bystander effects in tumour gene therapy are not general enough, it is necessary to design better vectors with a sustained potential to infect tumour cells. A virally infected cell becomes a factory of viral progeny and provides sustained amplification of the vector. Therefore, replicating viral vectors are attractive weapons in the arsenal of tumour warfare. Several excellent reviews are published on tumour-specific replicating viral vectors: (Yeung and Tufaro, 2000; Norman *et al.*, 2001; Wildner, 2001; Ring, 2002). Several viruses display natural selectivity for tumours, other viruses have to be mutated in order to limit their replication in normal cells.

Naturally occurring oncolytic viruses

Inherent tumour-selectivity is a characteristic of viruses as diverse as reovirus (a non-enveloped, double-stranded RNA virus), Vesicular stomatitis virus (VSV, enveloped, single-stranded RNA), Newcastle Disease Virus (NDV, negative-stranded, non-segmented RNA) and autonomous parvoviruses (non-enveloped, single-stranded DNA). By definition, naturally-occurring infections with these viruses are either asymptomatic (for example, reovirus) or cause relatively mild disease (for example, NDV).

Replication of RNA viruses is kept under control by double-stranded RNA induced protein kinase R (PKR), which phosphorylates the translation initiation factor eIF-2 α subunit and blocks translation initiation. Reovirus, VSV and Newcastle Disease Virus are thought to take advantage of the activated Ras pathway, which results in reduced PKR kinase activity, allowing virus replication to proceed. Ras-mediated signal transduction is activated in at least 30% of human cancers due to either mutated Ras or mutated/over-expressed epidermal growth factor receptor (Kirn *et al.*, 2001) (Norman *et al.*, 2001).

Vesicular Stomatitis Virus

VSV is efficient in replicating in carcinoma and melanoma cells, but not in primary fibroblasts. Intratumoural injections of the virus in nude mice inhibited tumour growth, however, some mice died of the infection. Treatment of tumour-bearing mice, who underwent experimental VSV viral therapy, with interferon, significantly reduced viral therapy mortality. Such an approach has the potential to be applied as an effective means of controlling viral therapy side-effects in the clinic, and increasing the therapeutic index of this potential oncolytic agent (Stojdl *et al.*, 2000a; Stojdl *et al.*, 2000b).

Reovirus

Reovirus is a small icosahedral non-enveloped virus, possessing an outer capsid with twelve vertices, surrounding an inner core containing the double-stranded RNA genome. Its genome is divided into ten segments of double-stranded RNA, which code for a total of eleven proteins. There are three size classes of RNA segments, designated L, M and S. It has been difficult to conclusively establish reovirus as an etiological agent for any disease. Reovirus has entered clinical trials; and because of its intrinsically benign nature and endogenous oncolytic properties, no modifications were

necessary. In mice, after multiple intratumoural injections of reovirus, complete responses were apparent in six of nine mice. Furthermore, no evidence of virus-induced toxicity was seen and mice remained tumour-free for at least 6 months following termination of the study. Reovirus-mediated tumour regression was possible regardless of whether the mice had previously been challenged by the virus and carried anti-reovirus antibodies (Norman *et al.*, 2001).

Autonomous parvoviruses

H1 and Minute Virus of Mice (MVM) show tumour selectivity in their replication. In normal cells, the single-stranded replicating genomes of autonomous parvoviruses induce p53 mediated block in cell-cycle and replication due to with free DNA ends. In tumour cells, replication proceeds and the cells are lysed. The non-structural proteins of H1 are involved in killing the cells (Telerman *et al.*, 1993).

Adapted viruses

Newcastle Disease Virus - an attenuated strain (PV701) has now been evaluated as an oncolytic agent in a Phase I dose-escalation trial of intravenous administration (approximately 70 patients). The most common adverse events were fever, chills, nausea/vomiting and fatigue; hypoxia and transient transaminasitis have been noted in patients with pulmonary or liver metastases respectively (Kirn *et al.*, 2001).

Measles virus - the live attenuated Edmonston-B vaccine strain of measles virus (MV-Edm) replicates selectively in human myeloma cells and has potent antitumour activity. *In vitro*, replication of MV-Edm was restricted in phytohemagglutinin (PHA)-stimulated peripheral blood lymphocytes (PBLs) but proceeded efficiently in a panel of 6 myeloma cell lines and in primary isolated myeloma cells, resulting in the formation of multinucleated syncytia and killing the cells. *In vivo*, when injected directly into ARH-77 myeloma xenografts in the mice, MV-Edm caused complete regression of these xenografts. MV-Edm administered intravenously into the tail veins of mice also showed significant antineoplastic activity against established RPMI 8226 and ARH-77 xenografts. In particular, the ARH-77 myeloma xenografts were exquisitely sensitive to MV-Edm therapy, and tumours in all mice regressed completely. (Grote *et al.*, 2001; Peng *et al.*, 2001)

Engineered viruses

Adenovirus: DNA viruses need to force cells into S-phase for productive replication, and suppress the G1 checkpoint mechanisms, mediated by p53 an pRb. The early viral antigens, such as large T of SV40, papillomavirus E6, and E1B55kD of adenovirus, target p53 tumour suppressor protein and inactivate it or target it for degradation. Large T targets both p53 and Rb. Adenovirus E1A binds pRb and releases the transcription factor E2F, which is repressed by pRb, which results in expression of genes involved in S-phase and chromatin synthesis, but also in adenovirus E2 region gene expression, allowing the viral replication to proceed. The G1 cell cycle check-point is dysregulated in all tumours, allowing them to replicate out of control. Therefore, a virus defective for its ability to force S-phase entry would not be able to replicate in normal cells – but could do so in tumours. This strategy has been used to

construct a number of different adenoviral vectors carrying larger or smaller deletions in E1A and E1B genes.

The first mutant constructed was the dl1520, deleted in E1B55kD, which binds p53. This conditionally replicating adenovirus is better known as Onyx 015. It allows selective replication in and lysis of p53-deficient tumour cells (Alemany *et al.*, 2000), and has shown preclinical efficacy against p53-deficient nude mouse-human ovarian carcinomatosis xenografts. The ONYX-015 virus has been used to treat carcinoma in phase I-III clinical trials for ovarian (Vasey *et al.*, 2002), head and neck (Vasey *et al.*, 2002), pancreatic (Mulvihill *et al.*, 2001) and colon (Reid *et al.*, 2001) cancer. Over 250 cancer patients have now been treated on approximately ten clinical trials. The virus was generally well tolerated at doses of up to 2 x 10(12) particles by intratumoural, intraperitoneal, hepatic arterial and i.v. administration; no maximally-tolerated doses were identified following intra-vascular administration. Viral replication was tumour-selective and was documented after administration by all routes; however, viral replication was variable depending on tumour histology. Single agent efficacy has been relatively limited to date (0-14% local tumour regression rates). In combination with chemotherapy, however, encouraging antitumour activity has been demonstrated (Kirn, 2001).

However, recent evidence points out that the oncolytic activity of the ONYX-015 virus is not strictly dependent of the cellular p53 status (Geoerger *et al.*, 2002; Petit *et al.*, 2002). Lack of ARF, an inhibitor of MDM2, can allow dl1520 replication in p53 positive cells, although exception to this rule exists as well (U2OS cells lack ARF and are non-permissive to dk1520 replication), demonstrating the complexity of the virus-cell interaction network.

E1B55kD has also other activities in addition to p53 binding, such as viral mRNA transport, which are impaired in the dl1520 and reduce the replication efficiency.

Moreover, p53 is reported to be mutated only in 50% of all tumours (although the p53 pathway is affected in a significantly larger proportion).

The pRb mediated cell cycle progression control is however, dysregulated in **all** tumours, and another adenovirus mutant, dl922-947 (D-24), deleted in E1A region binding to pRb demonstrated significantly greater potency than dl1520, and also, wild-type virus in some tumours, in a nude mouse human tumour xenograft model when administered intravenously (Heise *et al.*, 2000).

Herpesvirus

Mutant versions of HSV are made oncolytic by the deletion of two major genes, $\gamma_1$34.5 (encoding ICP34.5) and UL 39 (encoding the viral ribonucleotide reductase) (Martuza, 2000; Rampling *et al.*, 2000). The normal function of $\gamma_1$34.5 is to disrupt the cell's defense mechanism against infection, by targeting the PKR, a common defense mechanism against viral infection, as discussed above. It has also been suggested that association of ICP34.5 with the proliferating cell nuclear antigen (PCNA, involved in DNA replication and repair) could also enhance HSV-1 infection (Rampling *et al.*, 2000). As with reovirus, the Ras signaling pathway is the major determinant of host cell permissiveness to HSV-1 (Norman *et al.*, 2001). A second mutation in the UL 39 gene which encodes the large subunit of viral ribonucleotide reductase (or ICP6) (Mineta *et al.*, 1994; Mineta *et al.*, 1995; Hunter *et al.*, 1999) restricts the viral replication to cells having high levels of endogenous ribonucleotide reductase activity. Quiescent cells are protected. This virus is designated G207 and has been tested on animal models also for other than neurological types of cancer (colon,

ovarian, breast, prostate cancer and melanoma) (Chahlavi *et al.*, 1999; Coukos *et al.*, 2000b; Norman *et al.*, 2001; Blank *et al.*, 2002).
G207 and chemotherapy or radiotherapy has also been studied (Bradley *et al.*, 1999; Chahlavi *et al.*, 1999; Coukos *et al.*, 2000a). Cisplatin, which is routinely used for the treatment of squamous cell carcinoma, has been used in combination with G207 (Chahlavi *et al.*, 1999). In human cisplatin–sensitive tumours established in athymic mice, combination therapy has resulted in 100% cure rate in contrast to 42% with G207, or 14% with cisplatin alone, thus, the combined use is displaying synergistic effect. It is interesting to note that herpes therapy has also been shown to be effective against cancers that are resistant to chemotherapy (Coukos *et al.*, 2000a).

Poliovirus

Has been engineered to change its cell type specificity by inserting an IRES sequence from human rhinovirus 2. This replacement abolished neurovirulence in primates, but retained replication in glioblastoma cells. (Gromeier *et al.*, 2000). However, this substitution does not confer tumour-specificity, and the safety of such a recombinant virus must be considered seriously.

Transgenic oncolytic viruses

Immunoregulatory genes can be inserted into viruses, making them suitable for tumour immunotherapy. There is a large number of approaches to enhance tumour immunogenicity to levels where it is going to be rejected by the immune system. Many tumours are very poorly immunogenic, due to inefficient or aberrant antigen presentation, or lack of sufficiently mutant epitopes.

Vaccinia virus has an excellent safety record as a long-standing vaccine. Transgenes inserted into its thymidine kinase-encoding locus make the virus replicate preferentially in dividing cells, thus conferring some tumour-specificity. Vaccinia Virus carrying a GM-CSF gene is used for melanoma in phase I trial (Mastrangelo *et al.*, 1999; Mastrangelo *et al.*, 2000) and for bladder cancer (Gomella *et al.*, 2001).

HSV-1: Gene transfer of costimulatory ligands, such as soluble B7-1-Fc construct (Todo *et al*., 2001) or cytokines enhancing T-cell activation has been shown to facilitate tumour rejection by the immune system and in many cases provide immunity to challenge with the non-modified parental tumour.

HSV-tk is naturally present in herpesvirus constructs that are used for tumour therapy. This serves both a safety measure to control viral replication, but also gives a good possibility to target the tumour cells with the "conventional" suicide gene therapy.

Adenovirus carrying the Cytosine Deaminase-Thymidine Kinase (CD-TK) fusion gene in the E1B55kD locus has entered phase I clinical trials for prostate cancer (Kirn *et al.*, 2001). Also replication-deficient adenoviral vectors and retroviral vectors carrying HSV-tk gene have been used to treat tumours, but non-replicating vectors are not sufficiently efficient. (Sandmair *et al.*, 2000).

Virus-producing cells as vectors

Another approach to increase the efficiency of delivery of viral vectors to tumours is to inject the tumours not with virus, but with the virus producing cells. This approach has been used for treating glioblastomas with fibroblasts producing retroviral vectors carrying tk (Rainov, 2000), and neural precursor cells producing HSV-1 (rRp450) replicating only in dividing cells. The neural precursor cells are migratory

and showed strongly increased spread of virus compared to retrovirus-producing fibroblasts (Herrlinger *et al.*, 2000).

Bacterial vectors

Genetically modified, non-pathogenic bacteria can be used as potential antitumour agents, either to provide direct tumouricidal effects or to deliver tumouricidal or immunity-enhancing molecules. Attenuated Salmonella, Clostridium and Bifidobacterium are capable of multiplying selectively in tumours and inhibiting their growth in mouse models (Bermudes *et al.*, 2002).

However, the efficiency of natural attenuated Samonella in human clinical trials is not so great. An attenuated strain of Salmonella typhimurium (VNP20009), (deleted in purI and msbB genes), targets tumour and inhibits tumour growth in mice. In clinical phase I trial, for i.v. infusion of up to 10^9 PFU/m^2, for melanoma and renal carcinoma patients, tumour colonisation was observed in 3/24 patients, and none showed any tumour regression (Toso *et al.*, 2002).

Salmonella typhimurium, which expresses an *Escherichia coli* cytosine deaminase (CD), is currently undergoing phase I clinical trials in cancer patients (Bermudes *et al.*, 2002).

Also, Samonella can be used as an oral vector expressing CD40 ligand. It can protect mice against CD40+ B-cell lymphoma challenge by expression of CD40L in Peyer's patches' dendritic cells. Also, soluble CD40L was detected up to 8 weeks (Urashima *et al.*, 2000). Salmonella carrying melanoma antigen gp100 eukaryotic expression vector confers protection against melanoma in 70% of mice (Cochlovius *et al.*, 2002).

GENE THERAPY FOR GENETIC DISEASE

Several congenital genetic diseases result from a mutation of a single gene. If it was possible to replace the defective or missing gene with a "healthy" copy, the function of the cell would return to normal.

In patients with a hematological genetic defect, the type of defect determines which target cell, vector, and gene are used. Congenital immunodeficiencies have been the first study objects for gene therapy, because the molecular mechanisms underlying the conditions have been well characterized and there are relevant animal models available. In theory, all of the genetic defects treated by hematopoietic stem cell transplantation (HSCT) would benefit from gene transfer into HSC, whereas genetic defects treated by protein replacement would benefit from gene transfer *in vivo* into a cell able to export the synthesized protein.

Adenosine deaminase deficiency

Adenosine deaminase deficiency was the first disease to be treated using gene therapy because this gene was among the first to be identified. The adenosine deaminase enzyme is responsible for detoxification of metabolites in the purine salvage pathway. Four clinical trials have been conducted since 1990. The first protocol consisted of repeated infusions of peripheral T cells transduced ex vivo with an ADA retroviral vector (Blaese *et al.*, 1995). Of the other three clinical trials that targeted autologous HSC (Kohn *et al.*, 1995), (Hoogerbrugge *et al.*, 1996) (Bordignon *et al.*, 1995), only the study of Kohn et al. (Kohn *et al.*, 1995) showed unequivocally the selective advantage conferred *in vivo* by ADA transgene expression in T lymphocytes.

In fact, transgene-containing cells ranged from 1 to 10% in the T lymphocytes versus only 0.01% to 0.1% in the other hematopoietic lineages. Despite these encouraging results, a functional immune system was not restored because expression of the ADA gene was insufficient. Infants and children with ADA-deficient SCID have transgene-containing peripheral blood lymphocytes more than 7 years after treatment without suffering any adverse effects (Cavazzana-Calvo *et al.*, 2000), (Cavazzana-Calvo and Hacein-Bey-Abina, 2001) .

SCID-XI

SCID-XI is the first congenital genetic disease that has been successfully treated by gene therapy. It is caused by mutations in the γc encoding gene and accounts for 50 to 60% of patients with SCID. The γc chain is shared by several hematopoietic cytokine receptors including the IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 receptors (Malek *et al.*, 1999; Dumoutier *et al.*, 2000; Parrish-Novak *et al.*, 2000), which explains why T lymphocyte and natural killer cell lymphoid lineages are absent in this disease.

The first five patients lacking an HLA-identical donor were enrolled in a clinical trial in March 1999. The protocol consisted of marrow harvesting; CD34-cell purification; cell pre-activation and three cycles of transduction in a fibronectin-coated bag (Hacein-Bey *et al.*, 2001). Results for the first two treated patients were reported recently (Cavazzana-Calvo *et al.*, 2000). Transduced T-cells, and to some extent NK cells, developed within 3 to 4 months, which led to the development of both T-and B-cell antigen-specific responses. These two children are now living normally at home without any therapy. Similar results have now been observed in 2 of the other 3 children. A more than 2-year follow-up of these two patients has confirmed the preliminary data, which showed no adverse effects and a favorable clinical outcome. Sustained detection of thymopoiesis 1 year after treatment, as well as of a low percentage of transduced myeloid cells (in the order of 1‰), together with the presence of some transduced CD34(+) cells (Cavazzana-Calvo *et al.*, 2000), suggests that immature pluripotent progenitor cells have been transduced. The great difference in the frequencies of gene-modified cells in different lineages indicates a significant growth advantage of the gene-modified T-cells.

The potential next candidate diseases for gene therapy are the SCID conditions with an identical or closely related disease mechanism to SCID-X1, i.e., JAK-3 and IL-7Rα deficiency. SCID conditions caused by Rag-1, Rag-2, or Artemis gene mutations are also worthwhile to consider.

Reasons for the different success of ADA and SCID-X1 treatment

In ADA, the gene therapy did not result in full phenotypic correction of the disease, and the patients required continuing PEG-ADA infusions, whereas in SCID-X1, normal levels of immunity were restored and the patients responded normally to vaccinations. Three factors can potentially account for this. (1) All ADA-deficient patients received enzyme replacement therapy by weekly muscular injections of bovine ADA coupled to polyethylene (PEG-ADA) concomitantly with their gene therapy. This metabolically efficient treatment could have much reduced or even abrogated the survival advantage of transduced over nontransduced cells. (2) ADA deficiency appears to be a much more complex disease than the other SCID conditions. For instance, thymic epithelial cells are abnormal in ADA-deficient mice. It may well be that correction of the hematopoietic-derived cells will not provide full correction of the

immunodeficiency. (3) All clinical trials were performed in the early 90's. Many advances in gene transfer technology have occurred since, leading to at least a 10-fold gain in gene transfer efficiency, also, expression levels and stability of expression from the new retroviral constructs is far superior.

Other models for genetic disease

Chronic Granulomatous disease: Lentivirus vectors expressing gp91phox can partially correct the defect in model cell lines (Saulnier *et al.*, 2000). It is enough to correct the gene defect in a fraction of the stem cells, as it is known, that female carriers of X-CGD mutation can be phenotypically healthy with as little as 10% functional phagocytes in their circulation (Ochs HD, 1999). **Hemophilia**: In hemophilic dogs, intraportal injection of an AAV vector bearing a liver-specific promoter increased FIX levels to 4% (Wang *et al.*, 2000a). For FVIII deficiency, a single vector with a minigene expressing B-domain –deleted (BDD) FVIII has been constructed (Chao *et al.*, 2000). However, hemophilia gene therapy is hampered by antibody responses to the transgene (Balague *et al.*, 2000).

Promising results have been obtained as well in several other models of genetic disease – transfer of GDNF in **Parkinson's disease** (Bjorklund *et al.*, 2000; Bjorklund and Lindvall, 2000), **Duchenne muscular dystrophy** (Braun *et al.*, 2000), **mucopolysaccharidosis VII** (Bosch *et al.*, 2000a; Bosch *et al.*, 2000b), and **metachromatic leukodystrophy** (Consiglio *et al.*, 2001). For cell-based therapy of **type I diabetes**, a human cell line displaying regulated expression of insulin has been reported (Bailey *et al.*, 1999; MacFarlane *et al.*, 1999). Another promising approach for diabetes gene therapy relies on gene transfer of beta-cell specific transcription factor Pdx-1 to liver cells. Recombinant-adenovirus-mediated gene transfer of PDX-1 to the livers of BALB/C and C57BL/6 mice activates expression of the endogenous, otherwise silent, genes for mouse insulin 1 and 2 and prohormone convertase 1/3 (PC 1/3). Expression of insulin in this model ameliorates hyperglycemia in streptozotocin induced diabetes (Ferber *et al.*, 2000). These data indicate the capacity of PDX-1 transgene to reprogram liver tissue towards a beta-cell phenotype, and provides an example of a non-conventional approach to gene therapy.

GENE THERAPY FOR CANCER

The cancer can be attacked, using **1)** Conventional small-molecule drugs, which either damage DNA and induce apoptosis (chemotherapeutics), block signal transduction critical for tumour cell survival (Gleevec), or induce a conformational change in tumour-suppressor proteins, such as p53 (Prima-1) (Bykov *et al.*, 2002). **2)** Protein therapy, using antibodies, regulatory molecules, cytokines, immunogenic or antiangiogenic peptides, which destroy tumour's blood supply, can be injected. **3)** By gene therapy, using transfer of genetic material in the form of DNA, RNA or their synthetic analogues, using viral vectors to deliver the transgenes, or to take advantage of tumour specific genetic defects for their replication. Gene therapy for cancer is a vast and rapidly growing area of research, where recent years in particular have brought about several breakthroughs based on the understanding of molecular biology of cancer. We are focusing on the third approach.

Gene targeting

Although cancers generally contain multiple genetic alterations, mutations in a few common regulatory genes, known as oncogenes are responsible for malignant transformation and uncontrolled growth. As the result of the mutation is usually dominant gain of function effect, the therapeutic approach is to target these genes for down-regulation. It is possible to down-regulate translation from an mRNA by expression of a small RNA or transfer of a synthetic nucleic acid, usually modified DNA or PNA (peptide nucleic acid), complementary to the sequence (**antisense**) of the ribosome binding site of the target gene. Although the design of such antisense RNA molecules is largely intuitive and involves trial and error screening, several successful constructs have been developed for genes like Bcl-2, H-Ras, and N-Raf. More efficient than blockade of translation initiation is specific cleavage by **ribozymes** – small catalytic RNAs, which can sequence-specifically bind to their targets and cleave it – after which the RNA is degraded rapidly by endogenous nucleases. The genes successfully targeted in this manner include ErbR2, ErbB4, K-Ras, telomerase, c-Met and c-Myc. The result of such down-regulation is usually growth inhibition and sensitisation to chemotherapy, but also significant cell death in some cases like H-Ras (McCormick, 2001). This type of therapy is mainly limited to *in vitro* applications, due to short half-life of the antisense constructs and ribozymes *in vivo*, and difficulties with *in vivo* gene delivery to significant proportion of tumour cells. However, stable gene transfer of a ribozyme expression construct provides continuous expression and can be used for protection of cells from HIV-1 infection (Andang *et al.*, 1999; Maijgren-Steffensson *et al.*, 2001).

Gene transfer

By transferring a gene one can confer a new function to a cell. It is possible to modulate the tumour's immunogenicity, or to deliver a tumour-suppressor, angiostatic or suicide gene to the malignant cells or tumor stroma. Gene transfer to normal cells can serve the purpose of genetic marking of manipulated cells, or protection of cells form the toxic effects of chemotherapy, as in hematopoietic stem cell transplantation.

Immuno-gene therapy

The immune dysfunction in cancer is complex, involving specific and non-specific components, and can be mediated both by tumour cells and dysregulated non-malignant immune cells in a chronic inflammation type of reaction (Kiessling *et al.*, 1999). Approaches to overcome the problems in tumor immunology include tumor-antigen vaccination, enhanced immune presentation, and retargeting the cytotoxic effector functions by multifunctional chimeric proteins (Gidlof *et al.*, 1998; Totterman *et al.*, 1998; Ragnarsson *et al.*, 2001) of chimeric receptor gene transfer.

Since there are different molecular pathways for antigen presentation and immune effector function in MHC class I and class II-dependent antigen presentation and effector functions, the gene therapy approaches for these pathways can be viewed separately.

MHC class II dependent antigen presentation

In **classical vaccination**, antibody-mediated immunity is produced by administering protein combined with an adjuvant. The antigen is processed in antigen presenting cells (APC) and presented in MHC class II context. This method will generally not produce CD8+ cytotoxic T-cell responses. More efficient antigen presentation can be achieved by culturing the DC *in vitro*. *In vitro*, it is possible to

enhance class II antigen presentation by gene transfer of additional stimulatory molecules to the dendritic cells. Recombinant avipox (fowlpox, rF) vector that can efficiently express the transgenes for three human T-cell costimulatory molecules (B7-1, ICAM-1, and LFA-3) can transduce DC *in vitro* at high efficiency. The transduced DCs were shown to be more effective than only peptide-pulsed DCs in activating T cells to 9-mer peptides derived from two relatively weak "self" immunogens, i.e., human prostate-specific antigen and human carcinoembryonic antigen (Zhu *et al.*, 2001).

MHC class I dependent antigen presentation

To produce cytotoxic CD8+ **T-cell-mediated immunity**, expressed antigens will have to be presented on MHC class I complexes, and necessary costimulatory ligands have to be expressed on the cells presenting the antigenic peptide. Elimination of costimulatory signals is a major tumour strategy of avoiding the immune system and inducing, in fact, tolerance to the tumour. The strategies to overcome the tumour immune evasion and make the tumour immunogenic include expressed adjuvants, costimulatory molecules and cytokines, which stimulate the T-cells to produce a cytotoxic response to the tumour. It is also possible to perform efficient tumour therapy by genetically modifying the immune cells.

Genetic immunisation is performed in its simplest form by intramuscular injection of an eukaryotic expression construct as plasmid DNA. This will not lead to very efficient expression, and viral gene transfer is preferred to this method. The viral vectors suitable for this purpose are adenoviral vectors and RNA virus replicons – from Sindbis Virus and Semliki Forest Virus, which provide high level of expression and express viral proteins which might act as adjuvants for immune presentation. Vaccinia Virus vector containing the Human Papilloma Virus 16 (HPV-16)-E7 vaccine performed, on the other hand, worse than plasmid vaccine, due to inducing immunosuppressive IL-4 response (Chen *et al.*, 2001).

Adjuvants

Immunisation can be further enhanced by using specific adjuvants, which act only when fused directly to the antigen, presumably by targeting the antigen for more efficient presentation.

Several molecules have been characterised for their ability to potentiate antigen presentation to T-cells in a genetic immunisation model. The proteins used include Pseudomonas aeruginosa exotoxin A (ETA-dII) (Hung *et al.*, 2001b), tuberculosis bacterium heat-shock protein 70 (Hsu *et al.*, 2001), calreticulin (Cheng *et al.*, 2001a), lysosome targeting signals (Cheng *et al.*, 2001b), Marek's disease virus VP22 (Hung *et al.*, 2002), and HSV-1 VP22 (Cheng *et al.*, 2001c; Hung *et al.*, 2001a; Cheng *et al.*, 2002). These adjuvant proteins are proposed to function by distinct mechanisms – ETA-dII facilitates cell entry and escape from endosome, lysosome targeting signals produce protein retention in the lysosome, and VP22 proteins function by facilitating intercellular spread of the immunogen.

Expression of costimulatory molecules

B7-1 (CD80) expression on antigen-presenting cells and its counter-receptor CD28 on T lymphocytes plays a key role in the induction of cytotoxic CD8+ T-cell-mediated immune responses. Many tumours, including lung cancer, lack expression of B7-1 and this has been suggested to contribute to the failure of immune recognition of these diseases. When B7-1 expression is restored by adenoviral gene transfer, efficient

immune presentation ensues, with activity towards the non-modified, parental tumour (Iwakami *et al.*, 2001).

Efficient immune presentation can be achieved also using Herpes Simplex Virus amplicons expressing B7-1 or CD40L (CD154) in B-CLL. Interestingly, T-cell stimulation was dependent on the absence of the helper virus (Tolba *et al.*, 2001).
Expression of several stimulatory genes is usually better than a single gene. Adenoviral gene transfer of **CD154 and IL-12** to murine bladder cancer MB-49 cells completely eliminated the cells' tumourigenicity. Moreover, the mice were protected from parental tumour challenge (Loskog *et al.*, 2001). Also, IL-12 displays synergy with B7-1 in a myeloma cell model (Wen *et al.*, 2001).

Interestingly, B7-1 expression displays strong synergy with antivascular therapy. When EL-4 tumours were injected with B7-1 expression constructs, followed by systemically administered antitumour drugs 5,6-dimethylxanthenone-4-acetic acid (DMXAA) and flavone acetic acid (FAA), which cause tumour vasculature collapse and tumour necrosis, large (1 cm) and distant tumours regressed, and the cell mediated immunity was adoptively transferable, resulting in complete eradication. Single treatment was not effective (Kanwar *et al.*, 2001).

Expression of cytokines

GM-CSF is used for immune stimulation – In a phase I clinical trial, repeated vaccinations with irradiated autologous GM-CSF-producing melanoma tumour cells were well tolerated by patients and led to the activation of an antitumour immune response in some patients (Kusumoto *et al.*, 2001). Also, GM-CSF vector systems based on HSV and SFV are developed for tumour immunotherapy trials (Loudon *et al.*, 2001; Withoff *et al.*, 2001). But care must be taken – expression of GM-CSF in stomach can induce autoimmunity, with H+/K+-ATPase as the immunogen, in a transgenic mouse model (Biondo *et al.*, 2001). Still, this risk is usually accepted in cancer therapy. Immune stimulation combined with suicide gene therapy by using GM-CSF-IRES-TK combination, further enhances the opportunities of success (Guan *et al.*, 2001).

IL-2 Adenoviral gene transfer of IL-2 cDNA in established mastocytoma P815 model will lead to antitumour immunity and tumour reduction. This effect is correlated to the level of expression of IL-2 and importantly, can not be obtained by intratumoural injection of IL-2 protein. Also, the effect was abolished by preexisting immunity to adenovirus (Slos *et al.*, 2001). Combination transfer of IL-2 with the costimulatory ligand B7-1 by adenoviral vectors can induce an autologous antileukaemic response to B-CLL (Takahashi *et al.*, 2001).

Immune cell modification

Antigen presenting cells: It is possible to modify the antigen presenting cells, for more efficient presentation, or the immune effector cells, effectively bypassing the need for antigen-specific activation. DCs transduced with adenovirus expressing **IL-12** showed a high phagocytic ability similar to nontransduced DCs and were significantly superior to control DCs in the stimulation of autologous and allogeneic T lymphocyte responses (Nishimura *et al.*, 2001). DC can be gene-modified with the tumour antigen, and injected *in vivo*. The route of administration is of importance. A stable Dendritic Cell DC line that constitutively expresses the HPV-16 **E7** gene, generated the greatest anti-tumour immunity in a murine model by intramuscular administration, compared with subcutaneous and intravenous routes of administration (Wang *et al.*, 2000b). An

avipox virus vector expressing **B7-1 and CEA**, by transduction of DC *in vitro*, induced T-cell clones capable of lysing CEA expressing tumour cells (Tsang *et al.*, 2001).

Effector cells: Artificial chimeric receptors provide a promising approach to target T lymphocytes to tumour antigens. The receptors can produce either an activating, or a costimulatory signal, or both. A chimeric receptor, containing a scFv against prostate-specific membrane antigen (PSMA) and a combined T-cell receptor-zeta (TCRzeta) + CD28 signaling elements, deliver both the first and the costimulatory signal. Human primary T lymphocytes expressing these fusion receptors effectively lyse tumour cells expressing PSMA (Maher *et al.*, 2002).

Gene marking

Retrovirally transferred genes integrate into the chromosomes of the target cells and will be inherited by all the daughter cells of their progeny. The stability of this genetic modification and the ease and sensitivity of detection of specific genetic material by PCR enables to use retroviral gene marking to study the composition of the *in vitro* modified graft and trace the progeny of the individual modified cells.

Gene-marking studies were the first clinical protocols to introduce exogenous genetic material into human cells. Such studies provide information about normal cell biology and disease pathogenesis. Gene-marking studies have provided information about the biology of the human stem cell, factors that influence the efficiency of gene transfer, mechanisms of relapse after stem cell transplantation, and the pharmacodynamics of adoptive cellular immunotherapy (Bollard *et al.*, 2001). However, for many malignancies, marking efficiencies are insufficient for useful information to be obtained (Brenner, 1998). This is mainly related to the inability of MMLV-based retroviral vectors to transduce quiescent cells.

Marked malignant cells were found at the time of relapse in the majority of patients relapsing after autologous BMT for AML or neuroblastoma showing the infused marrow contributed to disease recurrence (Brenner and Rill, 1994; Brenner *et al.*, 1994; Heslop *et al.*, 1996).

Double gene-marking techniques with distinguishable retroviral vectors are being used to compare purging techniques and the reconstitution of different sources of stem cells (Heslop *et al.*, 1995). They also demonstrated the stability of gene expression from the stably integrated transgenes in the progeny of the HSC. The transgene could be detected up to three years in mature peripheral blood cells, both in lymphocytes and neutrophils, indicating the transduction of a true stem cell (Brenner *et al.*, 1993; Brenner *et al.*, 1996; Gahrton *et al.*, 1998).

Genetic protection

Genetic protection of cells from the toxic effects of chemotherapy is feasible by gene transfer. Several genes involved in DNA repair and drug metabolism have been identified, also proteins involved in pumping out foreign substances from cells.

Transduction of repopulating hematopoietic cells with a retroviral vector (SF1m) expressing the human multidrug resistance 1 gene (MDR1) protects the mice from toxic effects of paclitaxel. The dose chosen (8x20 mg/kg i.p. within 12 days) produced a non-lethal, reversible hematotoxicity in mice with steady-state hematopoiesis. Only 35.3% (6/17) of control mice survived when treated starting 14 days post BMT. In contrast, 83.3% (15/18) of mice transplanted with SF1m-transduced cells survived, owing to a significant protection against severe acute myelotoxicity (Carpinteiro *et al.*, 2002). MDR-1 also protects from etoposide (Schiedlmeier *et al.*, 2002). MDR-1 can be combined with gamma-glutamylcysteine synthetase (gamma-

GCS), the rate-limiting enzyme of glutathione biosynthesis (Rappa *et al.*, 2001) which provides protection from alkylating agents. Also, transfer of aldehyde dehydrogenase - 3 gene (ALDH-3) in combination with MDR-1 confers resistance to 4-hydroperoxy-cyclophosphamide, vincristine, and daunorubicin (Wang *et al.*, 2001).

Gene transfer of base excision repair proteins, formamidopyrimidine DNA glycosylase (FPG) or human oxoguanine DNA glycosylase (hOGG1), protects hematopoietic cells from N,N',N''-triethylenethiophosphoramide (thioTEPA)-induced toxicity *in vitro* and *in vivo* (Kobune *et al.*, 2001).

Tumour suppressor gene therapy

Tumour suppressor genes are usually inactivated or deleted in the course of tumourigenesis, therefore, the therapeutic approach is to deliver a functional copy of the gene to the tumour cells. The result is growth arrest and apoptosis, and also sensitisation to radio- or chemotherapy. The target genes are mainly those of well studied cell-cycle regulation proteins – pRb, p53, p14-ARF, p130, Ink4a and also Pten and Bcra-1 (McCormick, 2001). The vectors involved in this type of work are viral vectors with a good capability to deliver genes to tumour tissue, as well as the surrounding normal cells. Over-expression of tumour suppressor genes in normal tissue, however has no toxic effect. The function of the tumour suppressor proteins is regulated by the cellular environment and experiments have shown a 2-3 log therapeutic window in normal cells compared to tumour cells for p53 delivered by adenoviral vector (Adp53) to bronchial epithelial cells. (Zhang *et al.*, 1994). This vector is in phase II and III clinical trials for non-small-cell lung cancer and head and neck squamous cell carcinoma. It is well tolerated and has shown antitumour activity in a subset of patients, as well as synergy with chemotherapy and radiation. (Swisher *et al.*, 1999; Nemunaitis *et al.*, 2000)

Angiostatic gene therapy

Can be delivered by viral vectors to tumours, alone or in combination with other genes, to obtain a more widespread effect. At least 30 angiogenesis inhibitors, utilized alone or in combination with other therapeutic agents, are currently being tested in clinical trials in humans (Maio *et al.*, 2001). Since the endothelial cells are easily targeted, chemical gene delivery vehicles, like liposomal agents have proven effective in a mouse tumour model. Angiogenesis is an essential part of tumour formation, and has been shown to take place in all types of malignancies, including many hematological diseases (Moehler *et al.*, 2001). Angiostatic peptides are released by proteolytic cleavage from extracellular matrix proteins and take part in the natural regulation of wound healing and revascularisation. Angiostatin and endostatin (Sim *et al.*, 2000) attack the vascular endothelial cells in the growing blood vessel directly, however, they are not toxic to normal, existing blood vessels. Several strategies to suppress angiogenesis have been tested in animal models. Angiostatin and endostatin expressed from plasmid DNA complexed with liposomes inhibited growth of breast cancer in mice (Chen *et al.*, 1999). An adenovirus expressing secretable endostatin showed activity *in vitro* and in mouse models (Chen *et al.*, 2000), and a combination of viruses expressing three anti-angiogenic proteins led to complete tumour rejection in mouse models (Regulier *et al.*, 2001). Also, adenoviral expression of a soluble form of VEGF receptor was recently shown to suppress tumour growth in mouse models (Shiose *et al.*, 2000; Takayama *et al.*, 2000). The endothelial cells in tumours display several aberrant markers, and the blood vessels have abnormal structure and increased permeability. This is the result of the aberrant growth factor signalling in the tumour.

However, these endothelial cells are not genetically altered. Therefore, therapy targeting the normal cells which are necessary for tumour growth is not likely to be set back by developing resistance, a common problem with tumour chemotherapy.

Suicide gene therapy

Suicide gene therapy, in contrast to the previously discussed oncolytic viral therapy, involves controlled cell death induction in response to a prodrug. This is particularly important when cytotoxic immune cells are modified in the context of hematopoietic stem cell transplantation. The cells will have to be eliminated when complications arise. In the absence of complications, and the prodrug, the cells survive.

Suicide genes

A suicide gene is a gene, which can cause cell death. Therefore, all toxin genes would classify as suicide genes, causing unconditional cell death upon expression. The suicide genes that are useful in gene therapy, however, can cause cell death in response to certain chemical stimuli, so-called pro-drugs, which are non-toxic in themselves, but can be converted by the suicide gene product to toxic substances thus causing cell death. Table 3 displays the comon suicide genes. The characterised suicide genes originate mainly from viruses or bacteria. This can be a problem from immunological point of view, when the gene-modified cells can become a target for immune reaction. The only mammalian suicide gene systems are cytochromes and FKBP-Fas system. Also, different suicide gene systems display different levels of bystander effect. This is largely dependent on the nature of the converted prodrug. The prodrug is always an uncharged molecule, capable of crossing cell membranes and diffusing into the cells. After conversion, (e.g. phosphorylation) the charged molecule is not able to diffuse freely. Therefore, the bystander effect becomes dependent on intercellular communication channels.

FKBP-Fas: Fas (CD95, APO-1) is a member of the tumour necrosis–nerve growth factor receptor superfamily. Cross-linking of Fas results in the recruitment of a death-inducing signaling complex, activating a proteolytic cascade of caspases and inducing cell death by apoptosis (Ashkenazi and Dixit, 1998). Thomis and others have previously described a system for activating apoptosis at will and demonstrated its function in engineered cell lines (Rivera *et al.*, 1996; Amara *et al.*, 1997; Keenan *et al.*, 1998; Thomis *et al.*, 2001) using a chimeric protein containing the membrane-anchored intracellular domain of Fas fused to the FK506-binding protein, FKBP12. Cross-linking of these proteins by the addition of a bivalent FKBP ligand (a “dimerizer”) triggers the apoptotic death signal. Recently, a new dimerizer drug, designated AP1903, with specificity for the engineered FKBP over the endogenous protein has been developed and used for to control cell death by administration of the dimerizing drug (Thomis *et al.*, 2001).

The FKBP-Fas is probably not immunogenic. The only potential for immunogenicity in the construct are the point mutation in F36V-FKBP and 3 junction peptides. As a disadvantage, only 60% to 80% of Fas-engineered cells are eliminated by a single treatment of AP1903. The most recent retroviral vectors carrying the wild-type HSV-tk suicide gene are more efficient in killing highly proliferating cells — a single administration of the prodrug results in more than 90% T cell death *in vitro* (Tiberghien, 2001; Tiberghien *et al.*, 2001). However, by repeated exposure to the drug the Fas-engineered T cells can be eliminated nearly completely. On the other hand, killing of T cells expressing the Fas suicide gene is more rapid than that of cells

Table 3. Suicide genes (adapted from Dilber and Gahrton, 2001).

Gene	Prodrug	Active drug	GJIC indep. Bystander effect
Thymidine kinase (HSV-tk)	Ganciclovir	Ganciclovir triphosphate	-
Cytosine deaminase (CD)	5-flurocytosine (5-FC)	5-fluorouracil (5-FU)	+
Drosophila NK (Dm-dNK)	Bromovinyl-deouxyuridine	BVDU-phosphate	-
Guanosine-xanthine phosphoribosyl transferase (gpt)	6-Thioguanine (6-Tg)	6-Tg triphosphate	-
Purine nucleoside phosphorylase (PNP, DeoD)	6-MeP-dR	6-methylpurine	+
Nitroreductase (NTR)	CB1954	Hydroxylamine alkylating agents	+
CYP 2B1 (cytochrome p450)	Cyclophosphamide	4-hydroperoxy-cyclophosphamide	+
CYP 4B1 (cytochrome p450)	2-aminoanthracene (2-AA)	DNA alkylating agents	+
Varicella zoster virus tk (VZVtk)	AraM	AraM-MP	-
ß-glucosidase	Amygdalin	Cyanide	+
ß-lactamase	Vinca cephaloid	Vinca alk	+
FKBP-Fas	-	AP1903	-

expressing HSV-tk *in vitro*. Fas-engineered cells show signs of apoptosis within 1 hour of drug addition and killing is nearly complete after 24 hours, independent of the cell cycle status, whereas cell death induced by HSV-tk occurs over a time course of several days. The drug used to trigger suicide in the Fas system (AP1903) is a synthetic, small-molecule compound that does not interact with endogenous FKBP12(Thomis *et al.*, 2001). Such an independent system for induction of apoptosis may prove useful for suicide gene therapy applications where concomitant use of gancyclovir is necessary for treatment of viral disease.

Herpes simplex virus-1 thymidine kinase (HSV-tk) gene is one of the most commonly used and investigated suicide genes. Gancyclovir (GCV) is converted by the HSV-tk enzyme into a monophosphate form and subsequently to GCV-di and -triphosphate by endogenous mammalian kinases. GCV-triphosphate competes with normal nucleotides for DNA replication in mammalian cells and can cause cell growth inhibition and cell death. Also, incorporation of GCV into the nascent DNA strand will cause strand termination and induction of apoptosis by DNA-damage sensing mechanisms. Therefore, cells that express HSV-tk become sensitive to the toxic effect of GCV and can be eradicated *in vivo* by the administration of GCV.

Initial studies showed that tumour cells expressing HSV-tk can be killed *in vitro* or after reimplantation in mice in the presence of GCV. The HSV-tk enzyme is almost 1,000-fold more efficient at monophosphorylating GCV than endogenous thymidine kinase. Since phosphorylated GCV cannot pass a plasma membrane, it is accumulated within the cell. Suicide gene therapy can be used in combination with chemotherapy. In a mouse glioma tumour model, HSV-tk expression sensitizes tumour cells to chemotherapy with an alkylating agent temozolomide, which displays synergy with gancyclovir sensitivity (Rainov *et al.*, 2001).

Bystander effect and gap junction mediated intercellular communication

Gap junctions are important mediators of direct intercellular communication. Ions, small metabolite molecules, second messengers and certain dyes as well as excitational current can pass through gap junctions thus co-ordinating cellular activity and integrating cells in organs and tissues. Gap junctions consist of two hexameric integral membrane protein hemichannels termed connexons, which interact across the narrow extracellular space to create a complete channel (Unger *et al.*, 1997). The channels cluster together into macular domains called gap junctions.

Proteins forming connexons are called connexins (Cx). So far, 13 connexin genes have been cloned in rodents and 7 in man. Heterocellular gap junctions control cellular activity at organ level in brain (Cx43 in astrocytes, Cx32 in oligodendrocytes and some neurones) ovarium (Cx37) and serve as transport channels for metabolites (Cx26 in chorionic villi in placenta). Connexins are named by their molecular weight in kD deduced from their DNA sequence. Small molecule traffic between cells occurs by passive diffusion with a size limit of approximately 1000 D. Specificity of the intercellular channels for small molecules, as well as voltage dependent gating parameters, depend on connexin composition of the hemichannels. Intercellular communication via gap junctions is regulated at different levels (Musil *et al.*, 1990; Musil and Goodenough, 1990). Connexin proteins are stored intracellularly and transported to cytoplasmic membrane and assembled into gap junctions. These events are tightly regulated by differential phosphorylation by many protein kinases (Musil and Goodenough, 1991; Doble *et al.*, 2000). Protein kinase A activated by cAMP mediated signal is the only well characterised signal transduction system that increases gap junctional intercellular communication (GJIC) in most cell types (Flagg-Newton *et al.*, 1981). Traumatic events cause increase in Ca^{2+} and reduce pH, which may result in fast channel shutdown within minutes. Various tumour-promoting agents (TPA) and oxidative stress may cause GJIC shutdown and connexin internalisation (Hu *et al.*, 1995a; Hu *et al.*, 1995b).

Since gap junctions are important mediators of the bystander effect of HSV-tk cytotoxicity, many tumours lacking connexin expression are not susceptible to this form of treatment. GJIC downregulation has been shown to be an early step in tumour promotion. Mediators of inflammation (LPS, IL-1 beta and TNF alpha) are involved in downregulation of heterocellular, but not homocellular GJIC (Hu and Cotgreave, 1997).

Connexin43, the most studied connexin family member to date is the most widely expressed connexin in different cell types and the connexin that has been shown to mediate bystander killing effect (Dilber *et al.*, 1997; Touraine *et al.*, 1998a). Connexin43 expression has been studied in some tumour models of childhood malignancies. Studies in astrocytomas have shown that most low grade tumours are Cx43 positive and have functional gap junctions, whereas, in the high grade

glioblastomas the Cx43 expression is variable ((Shinoura *et al.*, 1996) and Asklund, manuscript submitted).

In vivo, the bystander effect is primarily dependent on gap junctions, but several additional factors can contribute to an enhancement in cell killing in several tumour models. Killing of endothelial cells in a tumour will result in occlusion of the blood vessels, thus bringing on the death of the tumour cells supplied by it. Immune system-mediated distant bystander effect, where non-gene modified tumours regressed together with the gene-modified ones, has been described in myeloma (Dilber *et al.*, 1996), head and neck (Bi *et al.*, 1997), hepatic (Kianmanesh *et al.*, 1997), colon (Agard *et al.*, 2001), and ovarial (Nagy *et al.*, 2000) cancer models. Viral gene transfer to tumours can stimulate the tumour-infiltrating immune cells to secrete cytokines, which can kill tumour cells, also, to induce immunity to the tumour. The distant effects can be vector-related - tk-Adenovirus + GCV mediated killing of tumours induces a potent antitumour immunity in a rat glioma model (Okada *et al.*, 2001). Other, transgene-dependent non-local effects have been described for p53-Adenovirus gene therapy (McCormick, 2001).

Since a significant fraction of malignant tumours are low expressers of Cx43 and display low gap junctional activity, it would be desirable to be able to upregulate tumour cell Cx43 expression. This can be done by carotenoid analogues (Zhang *et al.*, 1992), cytokines such as basic fibroblast growth factor (Doble and Kardami, 1995) and drugs like apigenin and lovastatin (Touraine *et al.*, 1998b).

Alternatively, it would be useful to bypass the requirement for gap junction formation - thus it would become possible to target tumours that do not express connexins. Proteins which can pass through cellular membranes can be used to construct fusions to therapeutic suicide proteins, such as HSV-tk.

Peptides for protein transfer

The first protein, which was discovered to translocate across cytoplasmic membranes in an energy-independent fashion, was the homeoprotein Antennapedia from Drosophila. Homeoproteins belong to a class of transactivating factors that bind to DNA through a specific sequence of 60 amino acids, the homeodomain. This latter sequence is structured three α-helices with one β-turn between helixes 2 and 3. Internalization experiments indicated that the homeodomain of Antennapedia is able to translocate across the cytoplasmic membrane of nerve cells by a non-energy-dependent mechanism and to reach the nucleus (Joliot *et al.*, 1991). A 16-amino acid long peptide, pAntp, corresponding to the third helix, is the minimal segment of the homeodomain capable of translocating through cell membranes (Derossi *et al.*, 1994). The internalization of pAntp is a receptor-independent process as indicated by the efficient cellular uptake of retro-, enantio-, and retro-inverso forms of this peptide (Derossi *et al.*, 1996).

However, pAntp cannot destabilize phospholipid membranes and cannot cross them at a low lipid-to-peptide molar ratio (Drin *et al.*, 2001). It is suggested that peptide-lipid interaction may only be the first step of a translocation mechanism which may involve other non-identified proteic components (Scheller *et al.*, 1999).

Synthetic fusion peptides to pAntp are taken up into the cells and retain their specific activity. This can be used for tumor therapy, e.g. to deliver mutant p53 reactivating C-terminal peptide 46 (Selivanova *et al.*, 1997).

Other basic amino acid-rich peptides such as transportan, (TP, a galanine-mastoparan chimeric peptide) (Pooga *et al.*, 1998a; Pooga *et al.*, 1998b; Pooga *et al.*,

1998c), TAT-derived peptides (Mann and Frankel, 1991; Vives *et al.*, 1997), and helical amphipathic model peptides are taken up by eukaryotic cells by a non-endocytic mechanism similar to those observed for pAntp. A recent structure-activity study indicated that the membrane-destabilizing propensities of designed helical amphipathic cell-permeable peptides cannot be correlated to their translocation activity. Recently it has been shown that amphipathicity is not really necessary for cell penetration, although it is necessary for retaining in the cell (Scheller *et al.*, 2000). It has been found, that various peptides with no similarities in primary nor secondary structure can translocate into cells – provided they are rich in arginine (Futaki *et al.*, 2001). The trnslocating peptides are summarised in table 4.

However, these protein vectors display several limitations - that they all require crosslinking to the target peptide or protein. Also, protein transduction using TAT fusion protein systems require denaturation of the protein before delivery to increase the accessibility of the TAT-PTD domain and decrease the resistance exerted by the protein conformation. A small peptide, pep-1 (21 aa) (Morris *et al.*, 2001) has been described recently. This peptide is designed from scratch, taking into consideration the conserved motifs of other protein transduction peptides. The sequence, KETWWETWWTEWSQPKKKRKV contains three domains: 1 – a hydrophobic tryptophan-rich motif, for targeting the cell membrane and forming hydrophobic interactions with other proteins, 2 – a hydrophilic lysine-rich domain derived from the NLS of the SV40 large T antigen, for intracellular delivery and solubility, and 3 - a spacer domain, SQP to improve flexibility and integrity of both domains. Tryptophan residues are conserved in several translocation peptides –pIsl (from LIM family homeoprotein Islet-1), pAntp, TP (Magzoub *et al.*, 2001) and VP22. Lysine- and arginine-rich motifs (Futaki *et al.*, 2001) are found in TAT, VP22 and several synthetic peptides (Scheller *et al.*, 2000). The pep-1 peptide is capable of forming stable complexes with other native proteins and transfer them to living cells, where they retain their specific activity (Morris *et al.*, 2001).

Table 4. Cell penetrating peptides.

Peptide	**Origin**	**Sequence**
pAntp (Joliot *et al.*, 1991)	Antennapedia	RQIKI**W**FQNRRMK**W**KK
pIsl (Magzoub *et al.*, 2001)	Islet-1	RVIRV**W**FQNKRCKDKK
TP (Pooga *et al.*, 1998a)	Galanin+ mastoparan	G**W**TLNSAGYLLGKINLKALALAALAKKIL
TAT (Vives *et al.*, 1997)	HIV-1 tat	YGRKKRRQRRR
Rex4-16 (Futaki *et al.*, 2001)	HTLV II rex	TRRQRTRRARRNR
I(Scheller *et al.*, 2000)	synthetic	KLALKLALKALKAALKLA
VP22 F81-195 (Aints *et al.*, 2001)	HSV-1	VPRTRRPVSGAVLSGPGPARAPPPPAGSGG AGRTPTTAPRAPRTQRVATKAPAAPAAET TRGRKSAQPESAALPDAPASTAPTRSKTPA QGLARKLHFSTAPPNPDAP**W**TPRVAG
Pep-1 (Morris *et al.*, 2001)	synthetic	KET**WW**ET**WW**TE**W**SQPKKKRKV

VP22

VP22, which is encoded by gene UL49 (Elliott and Meredith, 1992) is a basic, highly phosphorylated protein (Elliott *et al.*, 1996, 1999). VP22 protein has been shown to pass freely between cells by a unknown mechanism. VP22 can enter cultured cell, when added to growth medium (Elliott and O'Hare, 1997), like the peptides discussed before. However, endogenously synthesised VP22 can exit the cells and re-enter, a property, which makes VP22 useful in gene therapy applications. Thus, after endogenous synthesis in a small subpopulation of cells, the protein spreads and forms a gradient in surrounding recipient cells. In those cells, it concentrates in the nucleus and binds to chromatin. VP22 can spread to virtually every cell in a transfected monolayer from only a few producer cells (Elliott and O'Hare, 1997). VP22 fusion proteins can function after endogenous synthesis or exogenous application as a potent protein delivery system, making it a good candidate for efficient transfer of cytotoxic molecules and mediating bystander effect in the absence of gap junctions for cancer gene therapy.

VP22 as a protein vector

VP22 has been used for constructing fusion proteins to enable the expressed protein to spread between the target cells and enhance the desired effect. When VP22 is fused to p53 protein, the resulting chimera retains the ability to spread from transfected cells to nontransfected, p53-negative cells, and induce their apoptosis (Phelan *et al.*, 1998).

Also, VP22 fusion to herpesvirus thymidine kinase (VP22-TK) retains its prodrug conversion activity and is transferred to the surrounding cells in cell culture and *in vivo* in a mouse tumour model, in the absence of gap junction formation in a connexin negative tumour cell line. Gancyclovir treatment in this model system results in bystander effect and tumour eradication in the absence of GJIC (II).

VP22 mediated protein transfer can be utilised in vaccination schemes, where intracellular protein expression is utilised to promote antigen presentation via MHC class I pathway, in order to induce cytotoxic T-cell response. In plasmid mediated DNA immunisation schemes, VP22 fusion constructs show a significantly higher induction of specific CTL response against a model papillomavirus (HPV 16) E7 protein(Hung *et al.*, 2001a; Michel *et al.*, 2002). Also, when using alphavirus vector mediated transfer of RNA constructs for transient expression of immunogenic peptides, VP22-fusion constructs show significantly higher induction of specific CTL response(Cheng *et al.*, 2002). VP22 homologues from other herpesviruses, such as Bovine Herpesvirus improve immunogenicity in a similar manner(Oliveira *et al.*, 2001).

VP22 functional domains

VP22 mediated protein spread can deliver a significant amount of protein to virtually all cells present in the culture(Wybranietz *et al.*, 1999; Brewis *et al.*, 2000; Harms *et al.*, 2000). However, the functional activity of the transferred protein is lower than expected, in comparison to the amount detectable by immunostaining. The GFP-VP22 fusion protein is readily detectable in the surrounding cells by antibodies, although green fluorescence remains very low compared to adjacent transfected cells (I). Also, in the mouse tumour model, where mixtures of VP22-TK gene-modified and non-gene-modified cells were used, relatively high proportion (50%) of VP22-TK expressing cells was necessary to exert a full effect on tumour reduction in response to gancyclovir treatment(II). It is possible that in the process of translocation to the

recipient cell nucleus the fusion partners of VP22 suffer denaturation or, in the case of GFP, quenching, that is at least partially revertible by fixation. Therefore, we have analysed the VP22 protein in detail. Mapping of functional domains of the VP22 protein by deletion analysis and construction of fusions to GFP revealed several independent functional domains and localisation signals in the VP22 polypeptide. Two of the deletion constructs displayed capacity for intercellular transport and revealed an altered pattern of subcellular distribution in the recipient cells. These constructs can have a potential use in targeting defined functional peptides to specific subcellular locations (III).

SUICIDE GENE THERAPY IN HEMATOPOIETIC STEM CELL TRANSPLANTATION

Allogeneic hematopoietic stem cell transplantation (HSCT) is the treatment of choice for a range of malignant and non-malignant diseases. The first related transplants were performed in 1968 in two children with severe combined immunodeficiency and Wiskott-Aldrich syndrome. Recently, considerable progress in transplantation immunology has been made by introduction of advanced molecular methods of transplantation antigen typing. Improved clinical results have led to the use of hematopoietic transplantation at earlier stages of the natural history of several diseases. An HLA-matched sibling is available to fewer than 30% of patients. The number of unrelated HSCT is increasing and accounts for approximately 25% of the allogeneic transplants currently reported to the International Bone Marrow Transplant Registry (IBMTR) (de Lima and Champlin, 2001).

Suicide gene therapy can be used in HSCT for prevention of one of the common complications, graft versus host disease.

Sources of stem cells

Stem cells from different sources contain different amounts of mature immune effector cells, with different characteristics, which has practical consequences for engraftment and Graft-versus-Host Disease (GvHD).

Bone marrow is the location of hematopoietic stem cells in adults under normal conditions. Bone marrow is harvested under general anesthesia, from iliac bones. Up to 1 litre of marrow is usually collected. The majority of unrelated donor transplants use bone marrow as the source of stem cells.

The percentage of CD34+ cells among circulating total nucleated cells at steady state in healthy donors is 0.06%. In the BM it is 1.1%, an 18-fold difference in favor of the latter stem cell source.

Peripheral blood. Hematopoietic stem cells can be mobilized from the bone marrow by cytokines. When healthy donors are treated with recombinant human granulocyte colony-stimulating factor (rhG-CSF; 12 mg/kg per day) over 3 days, with another rhG-CSF dose given on the fourth day before the stem cell apheresis procedure, the mean peripheral blood (PB) CD34+ cell concentration increases from 3.8 x 10^9 /L to 61.9 x 10^9 /L, a 16.3-fold increase over baseline. The increase in early CD34+38- progenitor cells is, at 23.2-fold, even greater. In some protocols, GM-CSF and FLT3 ligand can be added. The stem cells are thereafter collected from the peripheral blood and enriched by apheresis The cell composition of unmanipulated PBSC and BM allo-grafts differs significantly. The total numbers of T cells, monocytes, and natural killer (NK) cells contained in a PBSC allograft are more than 10 times higher than those in a BM allograft, (Korbling and Anderlini, 2001) The cytokine mobilization and collection of peripheral blood cells from normal donors has

been extensively reviewed (Anderlini *et al.*, 1997). rhG-CSF mobilization treatment of healthy donors is safe and without any obvious adverse effects 3 years or longer after stem cell donation (Korbling and Anderlini, 2001).

Using PBSC, the incidence of acute GvHD appears to be similar to that observed with BMT, but the incidence of chronic GvHD may be increased. Faster hematological recovery and shorter initial admission to the hospital were observed with PBSC. (Champlin *et al*., 2000)

Cord blood contains adequate amounts of hematopoietic stem cells for stable engraftment in children. The immune effector cells in cord blood are immunologically naïve, therefore causing less GvHD. In pediatric patients, there is a decreased incidence of GvHD, and transplant results are comparable to those obtained with unrelated donor marrow transplants, allowing for greater HLA disparity (Rocha *et al.*, 2000; Barker *et al.*, 2001). Results in adults are less impressive, with problems related to delayed engraftment and defective immune reconstitution. Graft nucleated cell count is a major determinant of survival (de Lima and Champlin, 2001).

Possible complications

Complications following HSCT include graft failure, GvHD, infections, and relapse of the malignant disease.

Graft failure

Graft failure occurs usually due to rejection (incomplete conditioning), but infections or myelosuppressive drugs may also be the cause. Primary graft failure occurs when donor-derived hematopoiesis cannot be documented in the recipient after the transplant. Secondary graft failure is defined as a loss of donor-derived hematopoiesis after initial engraftment. Different definitions have been used, but the major diagnostic criterion involves failure to recover or maintain > 0.5 $x10^9$ neutrophils/L. The reported incidence of graft failure for matched unrelated donor bone marrow transplant (BMT) is in the range 3–11% (de Lima and Champlin, 2001). HLA incompatibility is a major determinant of graft rejection, mediated by immunologic mechanisms involving residual host-derived cells (Anasetti *et al.*, 1989; Anasetti and Hansen, 1994).

Graft-versus Host Disease

Graft-versus Host Disease occurs after transfer of immunocompetent cytotoxic cells to an immunocompromised individual who cannot reject them (Chao, 1999). The major determinants of GvHD are the HLA transplantation antigens, MHC class I and II. Minor histocompatibility antigens are peptides from other proteins presented by the MHC class I molecules. Important risk factors for the GvHD development include the number of immunocompetent cells in the graft, the level of HLA matching and the conditioning regimen of the recipient. Weak conditioning may result in graft rejection, and more importantly, relapse of the malignant disease, whereas intense conditioning has been shown to be one of the initiating factors for acute GvHD. (Goker *et al.*, 2001).

The course of GvHD

The course of GvHD can be divided into three phases, according to (Goker *et al.*, 2001).

***Phase1*:** Conditioning. Myeloablation with either total-body irradiation (TBI) or high-dose chemotherapy is used to cytoreduce underlying disease, and to suppress

host defense for preventing graft rejection. This can also cause extensive damage in host tissues including the intestinal mucosa, liver, and other tissues. Activated cells from damaged recipient tissues secrete many inflammatory cytokines, such as interleukin-1 (IL-1), tumour necrosis factor–alpha (TNF-a), granulocyte-macrophage colony-stimulating factor (GM-CSF), and interferon-g (IFN-g) (Xun *et al.*, 1994; Ferrara *et al.*, 1999). Dysregulated release of the cytokines may upregulate adhesion molecules and enhance recipient MHC antigen expression (Leeuwenberg *et al.*, 1988; Norton and Sloane, 1991; Hill *et al.*, 1997).

***Phase2*:** Induction and expansion. Presentation of recipient antigens to donor T cells, activation of donor T cells, and subsequent proliferation and differentiation of these activated T cells are crucial in this phase of acute GvHD. After the cellular component of the graft is infused, immunologically competent donor T cells recognize foreign host antigens presented by antigen presenting cells (APCs) in the context of MHC.

The response of the T-cells is determined by the costimulatory molecules: complete activation, partial activation, or anergy, i.e., a long-lasting state of antigen-specific unresponsiveness. The costimulatory molecules can be blocked by specific antibodies. The use of CTLA4-Ig to block B7:CD28 interaction to inhibit alloreactive donor T cells and induce a state of anergy has been shown in a clinical phase I trial (Guinan *et al.*, 1999). Also, alloreactive T-cells can be specifically eliminated in mixed lymphocyte culture by activation-induced cell death (AICD), mediated by CD95 antibody (Hartwig *et al.*, 2002).

Phase3:Effector phase. T-cells carry out the immune response by perforin, granzyme B, Fas-FasL interaction and cytokine secretion, e.g. TNF-α, IFN-γ, IL-2 etc.

Target organs for GvHD are mainly skin, liver and gut. *Skin* displays rash, which may progress to bullae, or even desquamation and necrolysis. *Liver* involvement displays as jaundice with conjugated hyperbilirubinemia and an elevated alkaline phosphatase concentration. This is due to damage to the bile canaliculi leading to cholestasis. *Gastroenteral system* GvHD may involve any location throughout the gastrointestinal (GI) tract. Diarrhea and abdominal cramping are generally the hallmarks of gut involvement. Clinical manifestations include nausea, vomiting, crampy abdominal pain, distention, paralytic ileus, intestinal bleeding, and voluminous, often bloody, diarrhea. *Hematolymphoid organs*: blood and lymph nodes. The compromised immune state may lead to frequent and serious infectious complications. GvHD may also affect hematopoiesis, and it may cause a reduction in peripheral blood counts.

GvHD prophylaxis

Immunosuppression with pharmacological agents is the main means to reduce the likelihood of GvHD. Immuno-suppressants, such as cyclosporine A (CsA), methotrexate (MTX), and corticosteroids are more effective when used in combination than as single agents. Tacrolimus (FK506), a macrolide antibiotic, although it is structurally unique, has a similar mechanism of action as CsA. It exerts its activity through binding to FK-binding protein. It has been found to be equally as effective as CsA Marrow T-cell depletion for GvHD prevention (Goker *et al.*, 2001).

T-cell depletion. One attractive method to prevent GvHD is to eliminate donor T lymphocytes. T-cell depletion (TCD) is an effective technique in preventing acute GvHD in murine models. TCD can be performed by the use of anti–T cell serum or monoclonal antibodies (either alone, with complement, or conjugated to toxins), such

as Campath-1 (anti-CD52, with very broad specificity, depleting T as well as other immune cells), antibodies targeting CD2 ± CD3 or CD5; or narrow-specificity antibodies, such as antibodies targeting T10B9 (/T-cell receptor) (Goker *et al.*, 2001) (de Lima and Champlin, 2001). Other techniques of T-cell depletion include lectins ±sheep red blood cells, and counterflow elutriation. Also, CD34+ cells can be positively selected by immunomagnetic means. TCD reduces the incidence of GvHD but has potential adverse effects. TCD adversely affects engraftment due to graft rejection by residual recipient T cells that survived the conditioning regimen. TCD also increases leukemic relapse, infections, and secondary malignancies. The result is no improvement in overall survival (Prentice *et al.*, 1984; Martin *et al.*, 1988; Chao, 1999). In a recent intriguing study of 70 patients who received HLA-matched allo-BMT, Campath-1G *in vivo* administration and Campath-1M ex vivo T cell depletion with no post-transplant prophylaxis was compared to historical IBMTR data, and acute GvHD was 4% in the Campath group vs 35% in the CsA+MTX group. Leukemia relapse at 5 years was 30% vs 29% (Hale *et al.*, 1998; Goker *et al.*, 2001). Interestingly, as resting NK cells express only low levels of CD52, these cells would have been present in the graft. NK cells have been suggested to mediate GVL effect, particularly in myeloid leukemias, but no GvHD (Ruggeri *et al.*, 2001; Martelli *et al.*, 2002; Ruggeri *et al.*, 2002). Since NK cell allorecognition mechanisms accept broader range of HLA antigens, the effect would be stronger in allogeneic, partially matched HSCT, as in haploidentical transplantation (Aversa *et al.*, 2001a; Aversa *et al.*, 2001b).

New agents for GvHD prevention include Mycophenolate Mofetil (an ester product of mycophenolic acid produced by *Penicillum sp*), Rapamycin (from *Streptomyces hygroscopicus*), trimetrexate (2,4-diaminoquinazoline folate analog), deoxyspergualin, chloroquine, PG27 (from *Tripterygium wilfordii* herb) CTLA4 and CD40L antibodies, GLAT (Glu-Lys-Ala-Tyr) and Neuraminidase (Goker *et al.*, 2001).

For treatment of GvHD, steroids are standard (methylprednisolone, 2 mg/kg/day), which can be complemented with ATG, CsA and antibodies to CD5, TNFα, IL-1R, IL-2R (Goker *et al.*, 2001).

Relapse

For effective immunological treatment for relapse after HSCT, donor lymphocyte infusions (DLI) are used. Also, DLI is an efficient treatment as adoptive immunotherapy for post-transplant EBV lymphoma. Donor lymphocytes can induce stable remissions in relapsing leukemia after HLA-identical sibling donor transplants. Up to 80% of patients with CML in cytogenetic or hematologic relapse in chronic phase may respond. The induction of remissions in this setting provides powerful *in vivo* proof of the existence of a graft-vs.-tumour effect. Responses to DLI have been documented in 20–45% of patients with relapsing AML and in 10–15% of subjects with relapsed ALL (Kolb *et al.*, 1995). Other diseases appear to be responsive, such as multiple myeloma, chronic lymphocytic leukemia and low-grade lymphomas. However, the treatment-related mortality may be as high as 30% and is usually related to GvHD, pancytopenia and immunosuppression-related complications. The experience with unrelated DLI is limited, but available data suggest similar efficacy and toxicity when compared to related donor lymphocytes (van Rhee *et al.*, 1998). A complete remission was achieved in 42% of cases. Grade II–IV acute GvHD developed in 25% of recipients; 41% developed chronic GvHD. Bone marrow aplasia developed in 12%(de Lima and Champlin, 2001).

Suicide gene modification for treatment of Graft-versus-Host Disease

In order to gain control over the transplanted T-cells in the case of GvHD, they can separated from the graft, cultured *in vitro* and modified by a suicide gene - a gene, which product induces cell death in response to specific external stimuli. The most widespread suicide gene is the herpes simplex virus-1 thymidine kinase gene (tk) (Verzeletti *et al.*, 1998) although other suicide genes have been proposed, namely equine herpesvirus tk (Loubiere *et al.*, 1999) and a synthetic suicide gene able to induce apoptosis via Fas pathway (Thomis *et al.*, 2001). HSV-TK phosphorylates a non-toxic nucleoside analogue, gancyclovir (GCV) to monophosphate, which is thereafter converted to toxic triphosphate by cellular kinases and incorporated into nascent strand of DNA. This results in termination of DNA synthesis and cell death. Administration of GCV will lead to elimination of only proliferating gene-modified cells, thus sparing the cells that are not reactive against alloantigens and mediating immunity against infectious agents.

In mouse model, it has been shown that GCV treatment is much more effective in preventing GvHD than curing an established disease (Cohen *et al.*, 1999a; Cohen *et al.*, 1999b). Most human trials with TK-modified T-cells have focused on delayed administration of GCV, with moderate success (Tiberghien, 1998, 2001). In a mouse model, treatment with GCV during the first 7 days after the transplant protected recipients from GvHD after grafting with marrow and TK-T cells. At day 60, survival in this group was 93%. The mice exhibited a normal immune response to viral challenge, also, when the donor mice had been immunized, the T-cell memory to viral antigens could be adoptively transferred (Cohen *et al.*, 2000).

It has to be pointed out, however, that in the aforementioned mouse experiments the grafts were collected from HSV-tk transgenic mice, thus there was no need for gene transfer and selection before the transplantation. In a clinical situation, the T-cells would have to be activated, transduced and selected. The cells that do not receive the transgene have to be eliminated, as these cells would not be responsive to suicide induction, an thus, uncontrollable in the case of GvHD. Selection marker genes are included in the gene transfer vectors and allow for selection of the gene-modified cells. The efficiency and efficacy of the marker gene are crucial for successful genetic modification of therapeutic cells.

MARKER GENES FOR CELL SELECTION

Marker genes serve the purpose of identifying the transgenic cells after gene transfer. While some biochemical markers, e.g. LacZ (β-galactosidase) and β-glucuronidase require usually fixation of the cells (although an *in vivo* fluorescent substance has been described for β-galactosidase) most marker genes serve the function of cell selection after gene transfer, in order to eliminate the non-modified cells.

Existing markers

Different marker genes for selection have been utilised, and these fall into two categories: metabolic selection markers and cell surface markers (Medin and Karlsson, 1997) for selection by FACS or immunomagnetic techniques. In general, metabolic selection markers allow for efficient background-free selection of the gene-modified cells, but the selection time is typically around one week. In some cases, the selection drug may be genotoxic (MDR-1). The cell surface markers allow the cells to be selected faster, but there is the danger of false positive selection if the selection is

performed too early, due to transfer of the marker protein in the retroviral envelope (Comoli *et al.*, 1996). The openness of the system leads to difficulties in maintaining sterility.

Table 5. Chemical markers (Adapted from Medin and Karlsson, 1997).

Marker	Approximate Size (aa)	Origin	Common Effector
OuaR	1020	Rat	Ouabain
LacZ	1020	*E.coli*	X-Gal
MDR-1	1280	Human	Taxol
NeoR	260	Bacterial	G418
HygR	350	Bacterial	Hygromycin
DHFR	190	Bacterial	Methotrexate
GPT	150	Bacterial	Aminopterin, Xanthine, Mycophenolic Acid
HisD	430	Bacterial	Histidinol

Table 6. Physical markers

Marker	Approximate Size (aa)	Origin	Function
CD34	320	Human	Blood cell progenitor antigen
NGFR	500	Human	Nerve growth factor receptor
GFP	240	Jellyfish	Fluorescence
CD18 (iβ2)	430	Human	Adhesion-dependent interactions
MurHSA	30	Mouse	Cell adhesion, costimulatory factor?
CD24	30	Human	Activation, oxidative burst?
IL-2R	270	Human	IL2 receptor α subunit
TfR	680	Human	Transferrin receptor
Leu-1	500	Mouse	Lymphocyte antigen
Thy-1	160	Mouse	Proliferation of blood progenitors?

Current marker genes utilised for cell labelling and selection include bacterial antibiotic resistance genes, e.g. neomycin phosphotransferase (Miller and Rosman, 1989) (NeoR) and others, multidrug resistance gene (Licht *et al.*, 1997) (MDR-1), ß-galactosidase (Nolan *et al.*, 1988) (LacZ), green fluorescent protein (Grignani *et al.*, 1998) (GFP), and membrane markers, such as truncated low affinity nerve growth factor receptor (Mavilio *et al.*, 1994) (ΔLNGFR), CD24 (Pawliuk *et al.*, 1994), murine Thy-1 (Planelles *et al.*, 1995), human CD4ζ, CD34 (Fehse *et al.*, 2000), CD19 and several others. Different markers can be utilised for different purposes, as each one has its advantages and downsides.

For cell selection in culture, bacterial resistance genes are useful, but slow (selection time about 1 week), and the markers are immunogenic *in vivo*. That will cause the expressing cells to be eliminated by the immune system in a few weeks after transfer. Also, NeoR will decrease expression of the IRES-linked partner in a bicistronic construct (Byun *et al.*, 1998).

Several recently developed transfer markers, such as LNGFR, rely on the detection of the marker protein on the cell membrane. Most of these are human proteins, which are not expressed naturally on the target cells. Fluorescent labelling by specific antibodies and separation by flow cytometry result in efficient selection (Fehse *et al.*, 1997). However, precautions must be taken to prevent false positive selection results using membrane markers. Expressed membrane proteins are included in the retroviral envelope membrane and are transferred to the target cell as the viral particle fuses to the cell membrane. Cytoplasmically deleted membrane proteins can be shed from the expressing cell into the culture medium, which can interfere with detection and reduce recovery in selection. The membrane markers have the most favourable characteristics from the immunological point of view, but the high rate of false positive selection results necessitates several time-consuming precautions such as sorting after 1 week of culture, which is often detrimental to stem cells. Sorting by FACS is inherently a slow process - which is a problem when large numbers of cells need to be transferred in a clinical setting. Maintenance of sterility is difficult in FACS and poses extra risks. Magnetic cell separation (MACS) is more feasible under these circumstances, but the risk for false positive selection remains topical. The existing antibody for labelling LNGFR gives a relatively high background and is expensive. Also, by MACS, only high-expressing cells are selected, which will result in significant loss of gene-modified cells.

Ouabain resistance gene (OuaR)

OuaR is rat Na+,K+-ATPase α1 carrying a L799C mutation that confers resistance to more than 1000 μM ouabain. This is 10^6 times higher than the human homologue. Na^+,K^+-ATPase is a housekeeping enzyme and present in all mammalian cells. It is important for cellular homeostasis of Na^+ and K^+. Utilising ATP as an energy source, it transfers 3Na+ ions to extracellular space and imports 2K+ ions per reaction cycle. The minimal functional enzyme active in the membrane is a heterotrimer of α, β and γ subunit. The α subunit contains the ion channels and ATPase domains. β and γ subunits serve a support function. The minimal functional enzyme active in the membrane is a dimer of α and β subunit. Four isoforms (α1, α2, α3 and α4) of Na^+,K^+-ATPase subunit gene family have been cloned so far in rat as well as in humans (Shull and Lingrel, 1986; Woo *et al.*, 1999), exhibiting different expression patterns in tissues, as well as different sensitivity to ouabain. The isoform α1 is the most resistant to cardiac glycosides (Lingrel *et al.*, 1998). Ouabain belongs to cardiac glycoside group of drugs. It is water soluble and acts within seconds. Ouabain binds to α subunit and inactivates its ATPase and ion-transport activity (Darnell *et al.*, 1995) which results in excess sodium in the cell. This is compensated by regulating the function of several other ion transporters, such as Na^+/Ca^{2+} exchanger, which results in increased intracellular Ca^{2+} levels and cell swelling. In cell culture, when ouabain is used to eliminate cells that lack the genetic modification of OuaR, blockade of Na/K exchange will lead to equilibration of intra- and extracellular sodium and potassium concentrations, and neutralisation of cell membrane potential. That will lead to dysfunction and leakage of mitochondria with the resulting stop of ATP production and induction of cell death.

OuaR has 96% amino acid sequence identity to the corresponding human Na+/K+ ATPase. Although as little as mutation of one amino acid can induce immune response, the high homology lends some hope that OuaR might be less immunogenic than bacterial resistance markers.

OuaR is useful in cell culture experiments and molecular biology for selection of human cells. Ouabain has been a clinically widely used cardiac medicine. It has no toxic effects apart from its action as an inhibitor of Na^+,K^--ATPase and it can be quickly removed by a simple wash. Its rapid course of action in the cell culture settings allows for quick selection of transiently transfected or stably transduced cells.

In vitro culture methods influencing T-cell function

Growth stimulation and expansion of cells is necessary not only for allowing transduction by MoMLV based vectors, but also to be able to transfer adequate numbers of cells to the patient. However, expanding cells for more than one week leads to skewing of T-cell receptor (TCR) repertoire (Ferrand *et al.*, 2000), inability of the cells to respond to mitogenic stimuli and loss of subsets of memory T-cells, most notably EBV-specific clones (Tiberghien, 2001), (Sauce *et al.*, 2002), (Carlens 2002, in press). Alternative protocol of stimulation, using CD3 and CD28 stimulation by monoclonal antibodies can prevent skewing of TCR subfamilies' representation (Dardalhon *et al.*, 2000), and activation induced cell death (Sauce *et al.*, 2002), but extensive *in vitro* expansion can lead the expanded cells close to the limit of their life span. A 1000-fold expansion requires approximately 10 cell population doublings on average (2^{10}=1024) which is a significant proportion of proliferative capacity for a mature T-cell. Moreover, not all cells in a culture survive, thus, the actual proliferation of the surviving cells can be expected to be even greater.

Therefore, we have tried to reduce the time requirement for T-cell selection to minimum, by using highly efficient and very rapid ouabain selection method.

AIMS

The overall aims of this study were to develop methods and vectors for gene delivery for genetic modification, and for suicide gene therapy in particular. The inherent limitations of today's gene delivery vehicles do not allow 100% efficiency of genetic modification of target cells, therefore, this shortcoming must be compensated by complementary techniques, as secondary delivery of the therapeutic protein from gene-modified cells to surrounding cells, or rapid and efficient removal of the non-modified cells.

The specific aims were

1. to characterise VP22 mediated protein spread from gene-modified cells
2. to study the efficacy of VP22 mediated suicide protein transfer in a mouse tumour model
3. to identify the determinants of spread in VP22 protein
4. to characterise a novel non-antibiotic selection system for human cell selection.
5. to study the efficacy of the OuaR selection marker for rapid selection of primary human T-cells.

METHODS

Characterisation of VP22 mediated spread (I-III)

The cell culture experiments for characterising protein localisation were carried out on several cell types to rule out cell line dependent peculiarities that might not give generalisable results. We used 293 and Cos-7 cells (displaying efficient gap junction mediated communication) as well as HeLa, C6 and Neuro2a cells, which display very low or no intercellular communication via gap junctions.

The question in the first study is the detectability of the VP22-fusion protein after intercellular spread. Therefore, we used two methods for detection: GFP autofluorescence, that is dependent on the native conformation of the GFP, and immunostaining with antibodies, which can detect the protein in both native and denatured forms. For GFP-VP22 detection, anti-GFP rabbit polyclonal antibody was used (Clontech) and secondary donkey-anti-rabbit Cy-3 conjugate antibody (Jackson Immuno-research Laboratories Inc, West Grove, PA) both according to the manufacturer's instructions. The cells were washed once in PBS, blocked for 30 min with BSA and incubated with the primary antibody in blocking solution for one h at a dilution suggested by the manufacturer, at room temperature, followed by three washes with PBS. The secondary antibody was diluted in the blocking solution and incubated for one h at room temperature, followed by three washes with PBS. Hoechst stain (Bisbenzimide H33342, Fluka Biochemica, Buchs, Switzerland) was used to counterstain the nuclei, at 1:4000 dilution in PBS for five min at room temperature followed by one PBS wash. Photographs were taken at 48 h post-transfection with a Leica DM RXA confocal microscope (Leica Microsystems Heidelberg Gmbh, Germany) and a Spectra Source Orbis digital camera (SpectraSource Instruments, Westlake Village, CA). Image processing was done using Slidebook 2.1.5 (Intelligent Imaging Innovations, Inc, Denver,CO) and Adobe Photoshop 5.0 (Adobe Systems Inc, Seattle, WA).

Analysis of VP22-mediated bystander effect *in vivo* (II)

C.B.-17/IcrHsd-Scid-beige mice (SCID-bg) were purchased from Harland Sprague–Dawley (Indianapolis, IN, USA). Mice were bred and maintained under specific-pathogen free conditions in the animal facilities of Huddinge Hospital and used at 5–8 weeks of age. All procedures involving animal use were approved by the local ethical committee. The SCID-bg mice have no functional T-, B-, and NK cells. This strain was chosen because of the possibility that the immune system may mediate the bystander effect.

The choice of the cell line for this experiment critical, since the bystander effect is strongly dependent on the GJIC. The Neuro2a cells display no GJIC, therefore, the bystander effect must be mediated byVP22, as the results showed as well.

The experiments on mouse tumours were performed as described previously by our group (Dilber *et al.*, 1997), namely, four tumours with different cellular mixtures were implanted on separate locations subcutaneously on the mouse back. This ensures that the differences in tumour sizes reflect the studied effect, not the variable reactions of the animal.

LNC-tk, LNC-VP22 or LNC-VP22-tk-transduced Neuro2A cells were mixed with LN-transduced Neuro 2A cells at different ratios as indicated in the text. Cells were injected s.c. into four separate locations (10^6 cells per site) on the back of each mouse: (1) 100% LN-transduced cells into the upper left; (2) 100% LNC-tk, LNC-VP22 or LNC-VP22-tk-transduced cells into the upper right; (3) 50% each of LN-transduced and LNC-tk, LNC-VP22 or LNC-VP22-tk-transduced cells into the lower left; (4) 75% LN-transduced and 25% LNC-tk, LNC-VP22 or LNC-VP22-tk transduced cells into the lower right location of the mice. Eight days after injection, those mice receiving a mixture of LN-transduced and LNC-tk, LNC-VP22 or LNC-VP22-tk-transduced cells were given 25 mg/kg GCV in 500 ml twice a day by i.p. injections. Mice in the control groups were injected i.p. with similar volumes of saline. Animals were examined at different time-points, and the size of tumours was measured using calipers. Animals were killed and dissected after 25 days. The tumour weight (mg) was estimated by calculating (length(mm) x (width (mm)2)/2.

Retroviral vector production (IV-V)

Retroviral vectors can be produced both transiently and from stable producer cell lines. Usually, small batches (2-5 ml) for functional testing are produced using transient transfection of Phoenix GP cells, and the vectors are pseudotyped with VSV-G envelope, which provides wide tropism for various cultured cell lines and physical stability of the vector. Then these batches can be used to transduce a second, stable packaging cell line for creating a stable producer.

Phoenix GP cells were transiently transfected with 4μg vector construct plasmid and 1μg VSV-G plasmid per 35 mm cell culture well. For transfections, Fugene 6 reagent (Roche Boehringer Mannheim, Germany) was used according to manufacturer's instructions. Briefly, DNA and Fugene reagent was complexed at 1/2 mass/volume ratio in 100μl volume of cell culture medium and added to cells after 15 minutes. Supernatant is collected 36 hours after transfection, filtered through 0.22μm Millex-GP syringe-top filter (Millipore Corporation, Bedford, MA) and used for transduction of PG13 packaging cell line. Titre was determined on HeLa cells and it was $4\text{-}5x10^5$ /ml.

The stable producer cells can be selected and cloned, in order to identify the producer clone with the highest titre. However, since PG13 cells are murine

fibroblasts, they are resistant to ouabain selection. Therefore we transduced the cells repeatedly, in order to obtain a cell pool producing high titres of virus.

PG13 packaging cell line was transduced with the retroviral vectors produced in the Phoenix GP cell line at four consecutive days at MOI 10 using 4 μg/ml polybrene. Polybrene is a positively charged polymer that facilitates virus-cell interactions by reducing the surface charges of the negatively charged particles. The transduced pools were expanded and retroviral vectors were collected according to the optimised protocol of (Eckert *et al.*, 2000), in 24h cycles in 100μl/cm^2 DMEM with 10% FCS. The supernatants were filtered through 0,2 μm Millipore Millex-GP filters and frozen at –80°C. The vectors were titrated on HeLa cells and had titres 4-5x10^5 per ml.

Characterisation of OuaR marker and ouabain selection (IV-V)

Quantification of cell survival during selection procedure with ouabain was performed with WST-1 reagent from Roche. This colorimetric reagent is oxidised by mitochondrial oxidases. In ouabain induced cell death both necrotic and apoptotic components can be detected (Xiao *et al.*, 2002), and this is largely dependent on the cell type studied. However, the first irreversible component of cell death in both cases is loss of mitochondrial function, due to acute ionic dysbalance in the cell. WST-1 is therefore more appropriate and faster-reacting than other widespread cell death analysis tools, e.g. Propidium Iodide staining of DNA. This method relies on cell membrane permeabilisation, which is known to be a late effect in cell death. Moreover, in apoptosis the cellular DNA is rapidly degraded; in many cases, particularly in primary cells, this leads to difficulties in adequate quantification.

Transduction and selection of primary human T-cells (IV-V)

Day 0. Mononuclear cells from healthy donor blood samples were separated on Lymphoprep (Axis-Shield PoC AS, Oslo, Norway) and cultured as described previously in (Carlens *et al*., 2000), in X-Vivo 15 (BioWhittaker, Walkersville, MD) supplemented with 5% human serum and 10 ng/ml anti-CD3 antibody OKT-3 (Ortho Biotech, Raritan, NJ) and 500 U/ml IL-2 (R&D Systems, Minneapolis, MN).

Day 3 and 4. The cells were transduced at MOI 3 using preloading technique - by spinning the culture plates containing the retroviral supernatant for 30 minutes at 1000g at 4° C. After that, the supernatant was removed and the T-cells were plated. After transduction, the cells were cultured in X-Vivo 15 with 5% human serum and 500U/ml IL-2.

Day 5. Ouabain, at 10μM final concentration was added to cell culture.

Day 7. Analysis: transduction efficiency, sensitivity to GCV and FACS analysis of the T-cell subsets.

RESULTS

Intercellular spread of GFP-VP22 in cell culture (I)

The intercellular spread of VP22 fusion proteins can be visualised after methanol fixation of cell cultures, and is not generally visible in living cells. After methanol fixation of the GFP-VP22 transfected cultures, the GFP fluorescence appears gradually in the recipient cells, during about five minutes after rehydration with PBS. Paraformaldehyde fixation crosslinks the proteins throughout the cell, thus ruling out the possibility of spread after fixation with organic solvents. However, when methanol is applied after paraformaldehyde, VP22-GFP protein becomes detectable in the cells surrounding the transfected cells by immunohistochemical detection. Autofluorescence of GFP is strongly reduced after transfer.

We conclude that it is possible to visualise VP22-mediated spread of proteins in the cell culture with proper fixation techniques. It is possible that in the process of translocation to the recipient cell nucleus the fusion partners of VP22 suffer denaturation or, in the case of GFP, quenching, that is at least partially revertible by fixation.

Intercellular delivery of functional thymidine kinase pro-drug activating enzyme, *in vitro* and *in vivo*, by the herpes simplex virus protein VP22 (II)

Both *in vitro* and *in vivo*, VP22 can be used to deliver functional tk protein from an expressing cell into many neighbouring cells, in the absence of gap junctions, where it functions in the conversion of the nucleoside analogue GCV to tri-phosphate GCV promoting cytotoxicity of dividing cells in culture and tumour regression *in vivo*. All animals receiving the 50:50 mixture of control LN-transduced and LNC-VP22-tk-transduced cells showed a significant tumour weight reduction at the site of inoculation following GCV administration. This result represents a significant difference between the tk alone and VP22-tk transduced cells in the reduction of admixed non-transduced cells (Figure 2).

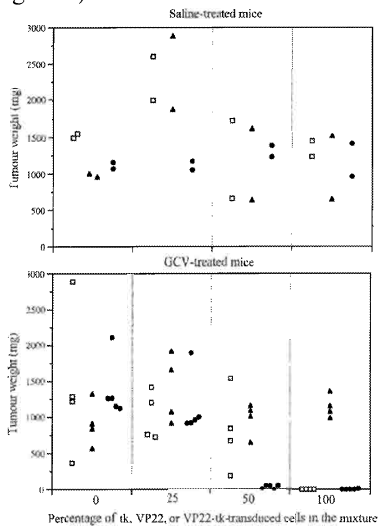


Figure 2. In vivo GCV-induced regression of tumour cells expressing VP22-tk. Bystander killing of Neuro 2A cells transduced with tk (square), VP22 (triangle), and VP22-tk (circle) in SCID-beige mice. **Upper panel**: saline-treated control mice; **lower panel**: GCV-treated mice. 10^6 Cells in different proportions of LNC-tk, LNC-VP22, LNC-VP22-tk transduced cells, to LN transduced control cells were injected into four separate locations on the back of each mouse. GCV treatment was started 8 days after injection.

These results demonstrate selective regression of tumours established with the 50:50 mixed population of VP22-tk transduced cells and represent a significant effect, which has not been observed with Neuro 2A cells expressing tk alone.

Mapping of herpes simplex virus-1 VP22 functional domains for inter-and subcellular protein targeting (III)

We have dissected the functional domains of VP22 by deleting selected portions of the molecule in GFP fusion constructs. Each of the deletion constructs displayed a different subcellular localisation pattern, a different effect on the cytoskeleton, and an altered intercellular transport capability. We have mapped the functions of VP22 to specific regions in the polypeptide as follows: intercellular transport - aa 81-195; binding and reorganisation of cytoskeleton - aa 159-267; nuclear targeting, inhibition of cytoskeleton collapse - aa 81-121; and nuclear targeting and facilitation of intercellular transport - aa 267-301 (Figure 3).

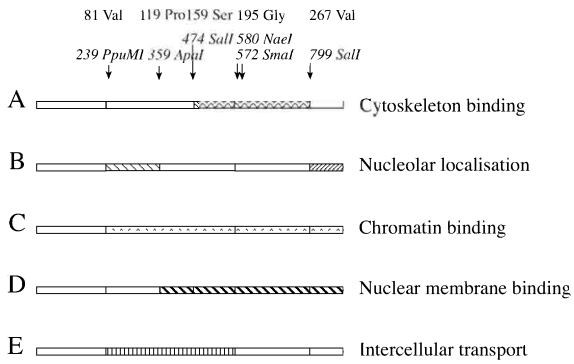


Figure 3. Locations of the functional domains in the VP22 polypeptide.

In conclusion, we have mapped the functions of VP22, which may be of benefit in protein delivery vector construction and the subcellular targeting of functional protein fusions.

Enhanced ouabain resistance gene as an eukaryotic selection marker (IV)

We have characterised an endogenous eukaryotic selection marker, ouabain resistance gene (OuaR), which has the potential for quick and efficient *in vitro* selection of target cells. The OuaR used by us is derived from the rat a1 isoform of Na+,K+-ATPase where leucine at position 799 is substituted for cysteine by targeted mutagenesis. This mutation confers resistance up to more than 1 mM ouabain *in vitro*. We show that cells transfected with plasmid (Figure 4) or transduced with a retrovirus vector encoding OuaR can be selected efficiently with ouabain in 48 hours and a pure population of cells can be obtained.

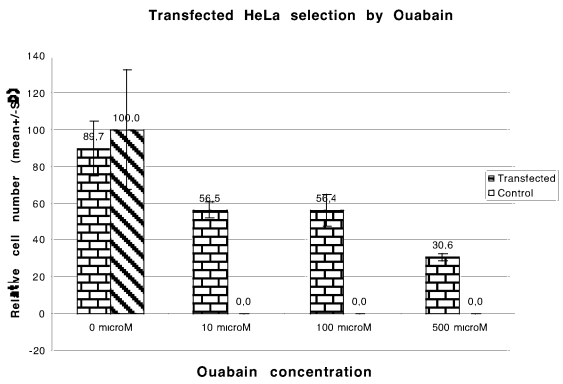


Figure 4. Selection of HeLa cells with ouabain after transient transfection. Selection medium was added to the cells 24 hours after transfection; 48 hours after transfection the cells were trypsinized, let to attach 12 hours, rinsed with PBS and quantitated with WST-1 cell proliferation reagent.

The ouabain resistance gene may be useful as a selection marker in general molecular biology, preclinical and clinical applications due to its short selection time and safety of ouabain for human use.

Rapid suicide gene transfer and selection of primary human T-cells (V)

We have prepared suicide gene therapy vectors containing HSV-tk and OuaR selection marker fusion gene in extensively optimised retroviral backbone SF91. The constructs contain structural elements for stable, high-level transgene expression in T-cells and transduce primary human T-cells at high efficiency. The OuaR selection marker allows the transduced cells to be selected chemically in a very short time – 36 hours, reducing the necessary time for *in vitro* culture to a week. The transduced T-cells display high sensitivity to gancyclovir. The rapid gene transfer and selection process prevents culture-related changes in T-cell function and helps to establish protocols to gain control over cytotoxic cells by suicide gene transfer.

CONCLUSION

To achieve successful results in genetic modification of cells, it is necessary to target a considerable fraction of the target cells. The efficiency of viral gene transfer using contemporary high titre vectors and optimised protocols can be as high as 90-95%. This high rate of genetic modification is good for many applications, but for several applications the rate of genetic modification of close to 100 % is necessary to achieve. Thus, the lack of gene transfer efficiency must be compensated by mechanisms, which take action after gene expression, that is, by properties of the expressed protein. It is possible to amplify the effect of the suicide gene by gap junctions, or by intercellular transfer of the protein mediated by VP22. VP22 mediated protein transfer can be used to deliver suicide proteins (TK) of marker genes (GFP) to specific subcellular localisations in the surrounding cell population. However, it is necessary to gene-modify a relatively high proportion (50%) of target cells for efficient transfer of functional protein.

In addition, it is possible to eliminate the non-modified cells rapidly by chemical selection. The speed of selection is crucial for many clinically relevant applications, e.g. T-cell transduction for preventing possible GvHD, and stem cell cultivation for genetic modification of hematopoietic stem cell graft. Selection by ouabain is the fastest method of chemical selection known to date and using OuaR as a selection marker for gene-modified cells it is possible to avoid the problems associated with extended *in vitro* culture of hematopoietic cells and achieve close to 100% gene-modified cell population ready for use.

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