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Gene Therapy

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The 2004 European Society of Gene Therapy (ESGT) meeting took place at Tampere Hall in Finland and highlighted advances in a variety of topics, including cancer, zinc-fingers, stem cells, small interfering RNA (siRNA), microRNA, and recent developments of non-viral and viral vectors. This meeting was attended by 513 participants from 32 countries, and included 106 oral and 224 poster presentations. One of the aims of this meeting was to take a critical look at gene therapy and the prospects for the future. Several presentations reported on RNA-based technologies, such as siRNA, as potential new classes of therapeutics against a wide range of diseases and for use in expression libraries to identify functional genes involved in biological phenotypes. Critical assessments were made of other aspects of gene therapy, such as genome editing and the use of protein transduction domains (PTDs) in gene- and protein-based therapies, where many researchers have failed to reproduce initial findings reported in the literature. Safety issues related to viral vectors were also important areas of discussion, especially following details released by the UK Gene Therapy Advisory Committee of perhaps the first known case of lentiviral vector-associated oncogenesis. Finally, updates were presented on the clinical development of viral vectors in anticancer therapies with evidence of significant improvements in the mean survival of patients.

Keywords: dsRNA, gene therapy, genome editing, protein transduction domains, miRNA, reducible polycations, RNAi, siRNA, smRNA, vectors

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1. Introduction

The 2004 European Society of Gene Therapy (ESGT) meeting took place at Tampere Hall in Finland and highlighted advances in a wide range of topics, including cancer, zinc-fingers, stem cells and recent developments of non-viral and viral vectors [101]. Of particular interest were a number of presentations highlighting the use of RNA molecules, such as small interfering RNA (siRNA), small modulatory RNA (smRNA) and ribozymes, to modulate gene expression. In addition, several speakers gave critical insights into other aspects of gene therapy, such as genome editing and the use of protein transduction domains (PTDs), where many research groups have failed to duplicate important findings previously reported in the literature.

Bernd Gänsbacher, president of the ESGT board, opened the conference with an excellent overview of the current state of gene therapy. Difficulties recently faced by the gene therapy community were highlighted, including adverse side effects in clinical trials with viral vectors, which have affected both the availability of funds to Biotech companies and the number of scientists working in this field. The number of gene therapy trials approved each year has also been reduced from 114 in 1999 to < 75 in 2003 [102]. There were signs of optimism, however, with patients still doing well in the French gene therapy trial for X-linked severe combined immunodeficiency.

It was also reported that the first gene therapy treatment had been licensed in China to Shenzhen SiBiono Gene Technologies Co. Ltd for head and neck squamous cell carcinoma for a treatment called Gendicine, an injectable medication that uses an adenoviral vector and p53 tumour suppressor gene. In addition, there are many other companies with products in the pipeline. B Gänsbacher finally announced that he would be stepping down as president of the ESGT board this year to be replaced by David Klatzmann (Paris, France).

The keynote lecture was presented by Inder Verma (The Salk Institute, California, USA), who provided a critical view on gene therapy and future prospects. Lentiviral vectors were identified as being close to reaching the requirements for an ideal vector system, particularly third-generation vectors that are fully functional with only 9.9% of the HIV genome remaining. There are still a few hurdles to overcome in developing these vectors for clinical use, such as effective regulation of gene expression and site-specific integration into the genome. A number of possible strategies to regulate gene expression were highlighted, including the use of tetracycline, zinc-fingers, RU-486, ecdysone and the SXR receptor. Many of these approaches, however, have a bacterial component and will need to be humanised to avoid eliciting an immune response. Finally, lentiviral vectors for non-gene therapy applications were reported, including stem cell research, transduction of embryos to generate transgenic animals, and the introduction of siRNA to silence gene expression in mice to study disease mechanisms. In particular, a lentiviral vector, termed lenti siGlut4, to downregulate expression of mGlut4 mRNA was described for studying mechanisms underlying insulin resistance.

2. RNA-based approaches

A wide range of therapeutic strategies for diseases such as cancer are being developed based on RNA molecules, such as siRNA, ribozymes and antisense RNA [1]. In particular, there has been considerable interest in double-stranded (ds) siRNA molecules that are typically 21 – 25 base pairs in length and silence gene expression by triggering RNA interference (RNAi) [2-3]. This is reflected by the increasing number of publications on RNAi that can be retrieved using the Web of Knowledge, which has risen from 6 in 1998, 124 in 2001 to > 1000 in 2004 [103]. The main challenges now lie in identifying the best strategy to introduce these types of molecules into cells and optimising the selection of dsRNA sequences targeted to each mRNA that can bring about effective RNAi [4].

M Kay (Department of Pediatrics and Genetics, Stanford, USA) presented data on the use of adeno-associated virus (AAV) vectors to express RNA molecules in the liver for the treatment of viral infections. Clinical trials using AAV vectors encoding blood coagulation Factor IX (FIX) for the treatment of haemophilia B demonstrated that hepatocytes can be transduced *in vivo* with 10 – 12% of the physiological levels of FIX activity in one patient at weeks 2 – 4 post vector infusion, although these levels fell back to pretreatment levels by week 12. Different pseudotypes of AAV were then evaluated in an attempt to overcome immune responses to this vector. In proof of principle experiments, severe toxicity problems were initially observed following the hydrodynamic delivery of AAV8-expressing siRNA directed towards hepatitis B virus (HBV) in a transgenic mouse model. However, the incorporation of a weaker promoter and a 19-mer siRNA molecule in this vector resulted in a 2.2 log reduction in HBV replication in the bloodstream that was still reduced after 5 months. A clinical trial to evaluate the AAV8-siRNA expression vector is now being planned for 2006.

R Agami (The Netherlands Cancer Institute, Netherlands) presented data on the use of a novel vector system, named pSUPER, to direct prolonged inhibition of gene expression in human somatic cells through RNAi. This system was used to specifically inactivate the oncogenic K-RAS12 allele, leading to a loss of tumourigenic phenotype in cancer cells, such as CAPAN-1 cells, that harbour this mutation. Novel tumour suppressor genes with RAS-like activity were then identified, including KLF4, ALG-2 and pituitary transcription factor 1 (PITX1), using a NK1 RNAi library. Investigation of PITX1 in primary human cells showed that induction of transformation by PITX required both the inhibition of p53 and p16. Reverse transcription-polymerase chain reaction (PCR) analysis further revealed the loss of PITX in colon cancer cells.

K Taira (Tokyo, Japan) gave an excellent presentation on the development of RNA molecules for use in therapeutic applications and the identification of genes involved in Fas-mediated apoptosis, Alzheimer's and metastasis. In particular, a novel strategy was described to trigger RNAi without induction of an interferon response by the introduction of 3 – 5 mutations within 20 nucleotides into the sense strand of long dsRNA molecules (> 25 bp). This approach should prove effective for silencing viral gene expression, as long dsRNA is processed in the cell to produce multiple siRNA molecules that would limit expression as a consequence of viral escape mutations. At present, the mechanism behind this method of gene silencing remains unclear and it appears to be suitable only for siRNA molecules expressed in the nucleus, but not for synthetic siRNA. Another strategy to silence gene expression was highlighted using siRNAs targeted to CpG islands within the promoter of specific genes [5]. Induction of transcriptional gene silencing occurred by means of DNA-methyltransferase-dependent methylation of DNA in human cells and this approach might have potential as a new type of gene therapeutic agent. Finally, the role of a small non-coding dsRNA, termed smRNA, in mediating neuronal differentiation was described, whereby the dsRNA triggers gene expression of neuron-specific genes through interaction with the transcriptional machinery [6]. It was proposed that alteration of neuronal gene expression was mediated through a dsRNA/protein interaction, rather than through siRNA- or miRNA-mediated gene silencing [7].

3. New technologies

Oligonucleotide-based systems, such as chimaeraplasts, have been reported with efficiencies as high as 20 – 50% of cells in mediating gene repair. M Blaease (Institute for Inherited Disease Research, Newtown, USA) gave a critical overview of gene editing technologies and described how, despite work by many laboratories, none of these highly positive reports have been reproducible. PCR artefacts caused by the carryover of the 'correcting oligonucleotide' have been found to be the explanation for many cases. Phenotypic readout systems using luciferase or green fluorescent protein (GFP) indicators showed that the activity of oligonucleotide-based systems were in the range of 1 in 10^3 – 1 in 10^5, rather than 20 – 50%. Chimeric endonucleases consisting of a synthetic zinc-finger DNA binding-domain coupled to a restriction enzyme (zinc-finger protein [ZFP]-nucleases) to target introduction of a double-strand break in DNA was highlighted as a promising approach to improve genome editing. Indeed, gene conversion rates of 4 – 15% were achieved using systems based on ZFP-nucleases. However, genome editing remains a tantalising prospect with many hurdles still to be overcome before it is ready for clinical use.

PTD proteins and peptides, such as the HIV transcription factor TAT and the herpes simplex virus protein VP22, have been reported to efficiently translocate the membrane of mammalian cells. This property has suggested a possible role of PTDs as vectors in gene- and protein-based therapies. M Johansson (Karolinska Institute, Stockholm, Sweden) gave a critical overview of PTDs and raised concerns that the membrane translocation properties of these proteins may be due to a fixation artefact. Strong adherence of PTD fusion proteins to cell surfaces was observed, but, following fixation of cells and tissues, the PTDs relocated to bind to nuclear DNA and other intracellular structures, giving the appearance of membrane translocation. Therefore, live microscopy of cells should be performed to study the properties of PTDs in order to avoid any potential fixation artefacts. Investigation of the mechanism of PTD membrane translocation suggested that the main property of both PTD peptides and PTD fusion proteins was to mediate cell surface adherence, and that internalisation of PTDs occurred by endocytosis. However, there was still some uncertainty concerning the precise role of PTDs, as in some studies, biological effects have been mediated by PTD fusion proteins.

T Eisenberger (Institute of Cell Biology, Witten, Germany) presented data on the development of a novel synthetic episomal vector for gene therapy that enabled safe and reproducible genetic modification of mammalian cells [8]. This vector contained a tetramer of a 172 bp minimal nuclear scaffold/matrix attachment region (S/MAR) linked to an upstream transcription unit, which was sufficient for episomal replication and mitotic stability of a mammalian episome. The ability of this vector to promote long-term expression of short hairpin RNA (shRNA) was also demonstrated, with > 95% reduction in expression levels using shRNA targeted to bcr-abl mRNA. An alternative strategy to support high-level transgene expression was described by N Mermod (University of Lausanne, Switzerland) using S/MAR elements to shield transgenes from chromatin inhibitory effects. In total, a collection of 10 short human elements enriched for S/MAR features were identified and cloned from genomic DNA. A significant improvement in the level of GFP-positive cells from 14 to 94% was observed following transfection when one of these S/MAR elements, termed huMAR 1-68, was incorporated upstream of an enhancer element in a GFP expression plasmid. Furthermore, high levels of gene expression with a huMAR-driven erythropoietin expression plasmid were demonstrated in mice following *in vivo* electroporation of the tibialis anterior muscle.

4. Vectors

A major hurdle to the therapeutic application of nucleic acids is a lack of suitable vectors and targeting ligands for their delivery. Synthetic vectors based on polycations or cationic lipids are promising vectors for gene delivery, as they are relatively safe, but the levels of gene expression mediated by these vectors are low compared with viral vectors. M Read (University of Birmingham, UK) reported on the development of a versatile synthetic vector capable of efficient delivery of a broad range of nucleic acids based on a reducible polycation (RPC) consisting of histidine and polylysine residues (HIS RPC). Previous studies had showed that RPCs based on the peptide $CK_{10}C$ facilitated efficient intracellular release of nucleic acids only in the presence of the endosomolytic agent chloroquine [10]. However, transfection experiments in a wide range of cell types using HIS RPC demonstrated superior gene transfer capabilities to 25-kDa polyethyleneimine (PEI), a gold standard synthetic vector, in the absence of chloroquine. In contrast to PEI, HIS RPC also mediated efficient transfer of different types of nucleic acids, including mRNA encoding for GFP in PC-3 cells and a 21-mer siRNA directed against the low-affinity neurotrophin receptor $p75^{NTR}$ in postmitotic dorsal root ganglion cell cultures. Furthermore, negligible toxicity was observed with HIS RPC compared with a 90% reduction in cellular viability when a fourfold lower dose of PEI was used. These findings indicated that HIS RPCs have properties useful for the delivery of nucleic acids that should enable the design and construction of efficient synthetic vectors for *in vivo* applications.

A Miller (IC-Vec Ltd, London, UK) described the rational design and development of self-assembly, synthetic complexes for DNA delivery involving cationic liposomes and the mu peptide. Despite significant development of these vectors, there still remains a number of hurdles, such as the nuclear barrier, that need to be overcome before clinically viable DNA delivery can be realised. Instead, these cationic liposome/lipid-based vector systems were better suited for the delivery of siRNA to cells. Several formulations to mediate siRNA

transfection were described [104], such as siFECTamine™ for *in vitro* applications, which brings about siFection at very low siRNA doses with negligible toxicity. Chemical modification to improve biological stability resulted in a formulation termed siFECTplus™ for *in vivo* applications, which gave 83% knockdown in gene expression by delivery of siRNA targeted to LacZ mRNA in mouse liver following hydrodynamic delivery of the α-galactosidase plasmid.

At present, *in vivo* gene transfer with AAV vectors can only be performed efficiently if the vector is administered directly to the desired organ or if the liver is the target. In an attempt to overcome this limitation, H Büning (University of Cologne, Germany) investigated the ability to manipulate the tropism of AAV vectors by inserting peptides (ligands) as large as 32 kDa into the viral capsid. The most frequently used sites for insertion within the AAV capsid proteins were amino acid positions 587 and 588. In most cases small peptides were selected by phage display, but larger inserts, such as immunoglobulin binding domains, were also used to enable coupling of cell-specific antibodies. AAV display technologies were also utilised to screen and identify AAV capsid mutants capable of targeting specific receptors. In particular, capsid mutants transduced target cells with up to 100-fold increased efficiency in a receptor-specific manner and without interacting with the primary receptor for wild-type AAV.

L Work and A Baker (University of Glasgow, UK) reported on the use of *in vivo* phage biopanning to isolate targeting peptides in spontaneously hypertensive stroke-prone and normotensive Wistar Kyoto rats. Intravenous infusion of a C7C-mer phage library, harvesting of organs and reinfusion over two subsequent rounds resulted in positive enrichment for phage homing to target organs. Increases in lung homing phage of 130- and 1700-fold were observed, for example, between rounds 1-2 and 2-3. Incorporation of these isolated peptides into the capsid of AAV2 after position 587 gave altered biodistribution profiles compared with control AAV. One of these peptides, termed Q, targeted AAV2 to the brain and lung, whereas another, termed V, targeted AAV2 to the lung and liver. Transduction via these targeted AAV vectors was shown to be independent of heparin.

5. Clinical trials

Primary brain tumours remain among the most lethal and difficult forms of cancer to treat. E Galun (Hadassah University Hospital, Jerusalem, Israel) presented data on a Phase I/II trial in patients with resistant glioblastoma to determine the safety and tumour response of repetitive intravenous administration of the non-engineered, oncolytic, lentogenic HUJ strain of Newcastle disease virus (NDV), termed OV001. Of the 14 patients enrolled (aged 11 – 58 years), 4/6 completed at least two cycles of 50 BIU units of OV001 (1 BIU = 1×10^9 50% egg infectious dose) during accelerated dose escalation, and 3/5 patients completed all three cycles of 10 BIU constant dosing. Toxicity was minimal, with grade I/II constitutional fever seen in five patients. The maximum dose was not achieved and anti-NDV haemagglutinin antibodies appeared within 5 – 29 days. An important finding in this early phase clinical trial was that one patient achieved a complete response.

M Vapalahti (University of Kuopio, Finland) described a Phase IIb study involving 36 patients to assess the safety and efficacy of Cerepro™ (EG-009, Ark Therapeutics Ltd), a replication-deficient adenoviral vector containing the herpes simplex thymidine kinase gene used in conjunction with ganciclovir for the treatment of patients with malignant glioma [105]. Cerepro treatment produced a clinically and statistically significant increase in mean survival from 39.0 ± 19.7 (standard deviation) to 70.6 ± 52.9 weeks ($p = 0.0095$). There was no evidence that the patients who lived longer and were treated with Cerepro had any deterioration in quality of life or an increased dependency on concomitant drug treatment. Six patients had increased anti-adenovirus antibody titres, without adverse effects. A multi-centre Phase III study is now being planned and should commence next year.

Finally, P Searle (University of Birmingham, UK) reported on the development of an anticancer strategy based on reduction of the prodrug 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954) to cytotoxic, DNA-crosslinking derivatives by the *Escherichia coli* nitroreductase (NTR) gene. Clinical trials to evaluate an adenoviral vector expressing the *NTR* gene (CTL102) in prostate and liver cancer patients showed that it was safe at all doses ($\leq 5 \times 10^{11}$ particles), and gave increasing levels and distribution of NTR expression as the dose was escalated. Instances of stable disease and post-treatment declines in α-fetoprotein or prostate-specific antigen were suggestive of tumouricidal activity. *In vitro* analysis of a conditionally replicating adenovirus (CRAd) expressing NTR showed higher expression and greater efficacy with CB1954 than a 20 times higher multiplicity of infection of CTL102. The catalytic activity of NTR with CB1954 was also improved by site-directed mutagenesis based on the crystal structure. In particular, one NTR mutant, F124K, termed '*turbo*-NTR', was ~ 5-fold more efficient than the wild type in sensitising cells to CB1954. This data supports the rationale for a future clinical trial of CRAd-*turbo*-NTR with CB1954.

6. Expert opinion

This conference covered a wide range of gene therapy technologies at various stages of development. In particular, RNA-based technologies, such as siRNA, represent a tremendous hope for new classes of therapeutics against a wide range of diseases. However, before siRNA-based technologies can reach their therapeutic potential, it will be necessary to evaluate their safety profile in more detail, as dose-dependent toxicity has been demonstrated. At this meeting it was highlighted that only a low dose of siRNA is required to trigger RNAi, as the RNA-induced silencing complex becomes saturated at concentrations of ≥ 20 nM. Off-target effects will most likely contribute towards toxicity with siRNA molecules, especially

if high doses and/or suboptimal dsRNA sequences are used. A recent study, for example, showed nonspecific effects of menin siRNA on p53 and p21 proteins, but not on actin. It was suggested that siRNAs can induce nonspecific effects on protein levels in an interferon-independent mechanism and this may be mediated by partial complementarity resulting in miRNA-like inhibition of translation [10]. Hence, to minimise off-target effects, specificity issues will need attention, as well as proper characterisation of gene silencing effects mediated by individual siRNA molecules.

Details were also released at this meeting by the UK Gene Therapy Advisory Committee (GTAC), as part of their horizon scanning activity, on a study demonstrating the development of liver tumours in a preclinical study using lentiviral vectors [106]. Most of the tumours occurred in a group of mice that had been treated *in utero* with a vector carrying the *FIX* gene, although a small number of tumours were also observed in animals that had been treated neonatally or had received vectors carrying only marker genes. This study highlighted by GTAC is perhaps the first known case of lentiviral vector-associated oncogenesis. As stated by the ESGT [101], it is premature to be concerned about the safety of lentiviral vectors until experiments have been performed to investigate the precise nature of the tumours and the mechanism of induction. Instead, this information should be useful to the gene therapy community to help focus research efforts to develop vectors that can avoid such problems and are suitable for clinical applications. The next ESGT meeting will be held in Prague, Czech Republic on 29th October – 1st November, 2005.

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