INVITED REVIEW

New perspectives for gene therapy in endocrinology

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Abstract

Gene therapy for endocrine diseases represents an exciting new type of molecular intervention that may be a curative one. Endocrine disorders that might be treated by gene therapy include monogenic diseases, such as GH deficiency and hypothalamic diabetes insipidus, and multifactorial diseases, such as diabetes mellitus, obesity and cancer. Premises seem promising for endocrine tumours, but many combined approaches of cell and gene therapy are foreseeable also for other endocrine disorders. This review outlines the principles of gene therapy, describes the endocrine disorders that might take advantage of gene transfer approaches, as well as the gene therapy interventions that have already been attempted, their major limitations and the problems that remain to be solved.

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Introduction

In the last three decades scientific progress in biomedical research has revealed the molecular mechanisms and genetic bases of many human diseases. The observation that some pathologies are caused by the inheritance of a single, functionally defective gene (monogenic diseases) generated the concept of gene-based therapy consisting of the supplementation of the defective gene with a functional one to be transferred to the affected cells.

Gene therapy, in its initial and broadest sense, can be defined as the transfer of a gene to a patient for therapeutic purposes. Initially developed as a strategy to treat inherited monogenic disorders by supplying the correct, wild-type copy of a mutated gene, gene therapy has gained importance as a tool to treat a growing number of human diseases. Indeed, the original concept of gene therapy as 'gene supplementation' has rapidly switched to the more general one of any strategy that employs genetic material (DNA or RNA) to prevent or cure a variety of diseases, many of which do not involve germ-line mutations, including multifactorial and somatic genetic diseases, such as diabetes mellitus, obesity, hypertension and cancer.

The development of more sophisticated techniques of molecular biology has allowed us to go rapidly from theory to application, as demonstrated by the 396 gene therapy clinical trials, involving 3278 patients worldwide, which have been undertaken since the first one

in 1989 (data from *Journal of Gene Medicine*, www.wiley.co.uk/genmed, updated to 1 September 1999) (1).

Even though the disorders targeted by gene therapy approaches span the entire spectrum of human diseases, as shown in Table 1 cancer has become a major interest, with 63.6% of the ongoing protocols involving cancer patients (1).

Notwithstanding the disappointing preliminary clinical results, gene therapy has become an established concept in medicine. The future success of gene therapy relies on the progress of the basic science, in the discovery of new disease-related genes, and in the development of suitable animal models of human diseases.

As for endocrine diseases, gene therapy is still at an experimental stage, since no clinical studies have been published so far. However, significant advances have been made during the last few years, and *in vitro* and *in vivo* studies suggest that several endocrine diseases could benefit from gene therapy.

Perspectives for gene therapy in endocrinology

Gene therapy strategies for endocrine diseases

Endocrine diseases can be divided into six broad categories, including subnormal hormone production, hormone overproduction, production of abnormal hormones, resistance to hormone action, abnormalities

Table 1 Update of gene therapy clinical trials.

	Protocols		Patients	
	Number	%	Number	%
Cancer	252	63.6	2269	69.2
Monogenic diseases	53	13.4	298	9.1
Gene marking	41	10.4	227	6.9
Infectious diseases	33	8.3	412	12.6
Healthy volunteers	2	0.5	6	0.2
Other	15	3.8	66	2
Total	396	100	3278	100

of hormone transport or metabolism, and multiple hormone abnormalities, including endocrine tumours (2). These disorders can result in either endocrine hyperfunction or hypofunction. Research progress in endocrinology has allowed the identification of inherited and acquired genetic disorders, as well as the molecular bases of many endocrine diseases, opening the possibility of correcting the underlying defect. Indeed, conventional treatments of most human disorders are often focused at improving symptoms, rather than at correcting the causative defects. Notwithstanding the success of current conventional therapy, the question arises as to whether there is a true need for gene therapy of endocrine diseases. A first answer could be that, in many cases, current therapeutic approaches cannot truly cure the patient, and that hormone replacement cannot always reproduce physiological hormone levels. Moreover, gene therapy may provide useful tools for the treatment of endocrine tumours and of immune-based endocrine disorders which do not respond to conventional treatments.

Different approaches of genetic intervention are foreseeable for endocrine diseases, as summarised in Fig. 1. Strategies for gene therapy of endocrine hyperfunction require either the transfer of a gene with ameliorating

effects, such as tyrosine hydroxylase in prolactinomas (3), or the inhibition of genes associated with hormone hypersecretion, by the use of antisense oligonucleotides or ribozymes. Endocrine hypofunction may require the delivery of the wild-type gene coding for the lacking hormone, such as the growth hormone (GH) gene in GH deficiency. Immunomodulating gene therapy could be useful in the treatment of autoimmune diseases that result in either endocrine hyperfunction or hypofunction. Benign and malignant endocrine tumours would take advantage of tumour suppressor gene delivery, oncogene inhibition, anti-angiogenic approaches or suicide and immunomodulating gene therapy.

Methods of gene transfer

Gene therapy requires efficient delivery of nucleic acid sequences to target cells, adequate and prolonged expression of the introduced gene, without any toxic side-effects for the target tissue and for the individual as a whole. *Ex vivo* and *in vivo* approaches have been used. *Ex vivo* strategies require the removal of cells from the body, their transduction by viral or non-viral vectors carrying the therapeutic gene, and their reinfusion into the patient. The *ex vivo* approach has the advantage that safety of modified cells can be evaluated before their administration to the patient. However, isolation of a sufficient number of cells and their appropriate manipulation in culture without causing undesirable changes in their biological properties are required. In the *in vivo* approach the vector carrying the therapeutic gene is directly administered to the patient.

Given the identification and availability of a large number of therapeutic sequences, the limiting step for a successful gene therapy is the development of an efficient gene transfer system to reach the target cells. With few exceptions, such as the skeletal muscle and the skin, naked DNA is not efficiently taken up

Figure 1 Gene therapy approaches for endocrine diseases.

Table 2 Main features of the most commonly used vector systems.

	Retrovirus	Adenovirus	HSV	AAV	Liposomes	Naked DNA
Insert size	8 kb	35 kb	>20 kb	<4 kb	>20 kb	>20 kb
Functional titre (cfu/ml)	10 ⁷	10 ¹¹	10 ¹⁰	10 ⁹	Not defined	Not defined
Integration	Yes	No	No	Unsure	No	No
Sustained expression	Variable	Transient	Transient	Variable	Transient	Transient
<i>In vivo</i> delivery	Poor	High	High	High	Variable	Muscular tissue
Quiescent cells	Only lentivirus	Yes	Yes	Yes	Yes	Yes

and expressed by mammalian cells. For this reason it is necessary to design suitable vectors for gene transfer and gene expression. Most vectors presently in use are based upon viruses, such as retroviruses, adenoviruses, adeno-associated viruses (AAVs) and herpes viruses.

The wild-type virus is genetically modified to be unable to replicate and to be non-pathogenic, while retaining infectivity. Each viral vector presents advantages and disadvantages; as a group, viral vectors still cause much concern about safety. Non-viral vectors, such as liposomes and molecular conjugates, partially overcome this last issue, but remain much less efficient than viral vectors (Table 2).

The choice of a vector is dependent upon the target cell. In gene therapy approaches for rapidly proliferating cells, such as cancer cells, retroviral vectors are the most indicated, since they only infect dividing cells. If the target cells are quiescent, adenoviral and AAV vectors are the most suitable ones, since they infect both dividing and non-dividing cells. Another factor to be considered in choosing a vector is the size of the therapeutic gene that has to be transferred. Adenoviral vectors can accommodate up to 30 kb of foreign genes, whereas AAV vectors no more than 4.5 kb. Retroviral vectors have a gene transfer capacity of about 7–8 kb.

When a long-term expression of the therapeutic cassette is required, retroviral vectors are the most appealing ones, since they integrate into the host genome, assuring a quite stable expression. On the contrary, with adenoviral vectors, whose genome is carried extra-chromosomally, repeated administrations are necessary to obtain a sustained expression. The immune response against adenoviral vectors, which is driven by the expression of viral proteins, and the need for repeated administrations, can be deleterious in those cases when stable transgene expression is required; in some others, as in cancer gene therapy, it may prove useful, helping in tumour recognition and killing.

A major limitation of the gene transfer systems so far developed is the difficulty in obtaining clinically relevant levels of gene expression. Moreover, no single vector has emerged as being optimal for all applications. Current systems differ in their suitability for *ex vivo* versus *in vivo* adoption, their capability for persistent gene expression, and for the induction of toxicity. Each of the different vector systems presents advantages and disadvantages, which depend also on

the disease that has to be treated. An ideal vector containing all the possible components to overcome the barriers to gene delivery is depicted in Fig. 2.

Targeted gene therapy in endocrinology

An important issue in the development of gene therapy protocols is the need to target therapeutic gene delivery. Indeed, safety is a primary concern of gene therapy, and targeted vectors are required both to minimise the risk of germ-line cell transduction and to prevent side-effects to the surrounding healthy tissues. Moreover, targeting can reduce vector wastage, and the amount of vector stocks that need to be produced and administered *in vivo* in order to achieve therapeutic levels of transduction.

Targeting of vectors can be obtained in many ways. The easiest one is to deliver the vector directly at the target site. For systemic administration molecular engineering is required to target either gene expression or gene delivery. A transcriptional targeting can be attempted by the introduction of tissue-specific or tumour-specific enhancers/promoters to control the expression of the therapeutic genes. Targeted gene delivery may be achieved also by the exploitation of the natural tropism shown by some viruses for specific tissues (e.g. hepatitis virus for liver cells), or by modification of the viral tropism (viral envelope pseudo-typing; expression of antibodies or ligands on the viral particle surface to confer new binding specificity towards target cell receptors).

Endocrine glands appear to favour targeted gene therapy at different levels: (i) the easily accessible anatomical site of some endocrine glands (thyroid, pituitary) allows the direct inoculation of the vector, and the evaluation of cell transduction; and (ii) transcriptional control elements (enhancer/promoter) for the expression of tissue-specific genes (hormones, hormone receptors) may be employed to selectively direct transgene expression (Table 3); and (iii) tissue-specific surface proteins (such as hormone receptors) may be used as targets for vectors with modified tropism.

Targeted gene correction in endocrinology

Gene therapy has been traditionally conceived as 'gene supplementation', an approach in which the

Figure 2 The ideal vector containing hypothetical components to overcome the barriers to gene delivery.

deficiency/dysfunction of a protein is corrected by the introduction of a copy of the wild-type gene that codes for that protein into the affected cells. Indeed, all studies of gene therapy for endocrine diseases published so far,

Table 3 Examples of endocrine-specific promoters/enhancers suitable for the development of transcriptionally targeted gene therapy.

Cell type	Specific promoter/enhancer
Pituitary	
PRL-secreting	PRL
GH-secreting	GH
TSH-secreting	β -Subunit of TSH
FSH/LH-secreting	β -Subunit of FSH/LH
ACTH-secreting	Pro-opiomelanocortin
Non-secreting	α -Subunit of glycoprotein hormones
Thyroid	
Follicular	TSH receptor Thyroglobulin Thyroxine oxidase Sodium/iodide symporter
Parafollicular (C cell)	Calcitonin/calcitonin gene-related peptide
Parathyroid	Parathyroid hormone
Adrenal cortex	CYP11B1 CYP11B2 ACTH receptor
Endocrine pancreas	
β -Cell	Proinsulin
α -Cell	Proglucagon
Neuroendocrine cells	Chromogranin A Somatostatin receptors

TSH = thyrotrophin; FSH = follicle-stimulating hormone; LH = luteinising hormone.

have employed a gene supplementation approach. However, this strategy has some limits: (i) the gene is often too large and has complex regulatory sequences, so that it cannot be introduced into a vector system; (ii) in some cases, host immune responses against the viral vector reduce transfer efficiency and preclude repeated administration; (iii) some genes require a tightly regulated expression, so that achieving their balanced and functional expression becomes challenging; and (iv) it is difficult to deliver enough vector to the target cells to obtain a therapeutic effect.

An alternative approach is to correct the genetic defect by targeting a dysfunctional exon, or the whole gene, directly substituting it with a correct copy by homologous recombination. The low frequency of homologous recombination in mammalian cells and the possibility of random insertion in the absence of sequence homology are major limitations to this approach (4). A novel technique, which exploits the DNA repair system of the cell, has recently been developed to correct genetic diseases that result from single-point mutations. This technique exploits the possibility of introducing site-specific changes in genomic DNA using oligonucleotides (5). The molecules can be chemically modified so that, upon pairing with the target sequence in the DNA, their chemically reactive group is activated and produces a modification in the genome (6). In this regard, RNA-DNA chimeric oligonucleotides have been designed to contain a sequence complementary to that of the target gene except for a single mismatched nucleotide. The unpaired nucleotide is apparently recognised by endogenous mismatch repair systems, thus altering the sequence of the target gene. This strategy has been successfully used to correct

point mutations of a variety of genes, including human alkaline phosphatase (7, 8), β -globin (9), factor IX (10) and UDP-glucuronosyltransferase-1 genes (11). It is conceivable that, in the future, a similar approach could be exploitable in a large number of monogenic diseases resulting from single-point mutations, including endocrine diseases, such as congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency.

Corrective gene therapy for monogenic endocrine diseases

The first disease models for which gene therapy has been envisioned are those resulting from a single gene mutation that can be corrected by supplementing the functional gene. For many endocrine disorders, however, at variance with most inborn errors of metabolism (12), gene therapy should restore not only the missing gene product, but also the intrinsic, strict regulation of native gene expression. Indeed, most hormones are secreted in a pulsatile fashion and show circadian rhythms. Regulated hormone delivery is essential to achieve optimal physiological effects.

Familial hypercholesterolaemia (FH)

FH has been one of the first models of monogenic diseases for the development of human gene therapy. This metabolic disorder, caused by the inherited deficiency of the low density lipoprotein (LDL) receptor, is associated with severe hypercholesterolaemia and premature coronary artery disease. The homozygous form of FH is a good candidate for gene therapy, since it is a lethal disorder refractory to other treatments, such as LDL removal by apheresis, or orthotopic liver transplantation. The first gene therapy approach developed for FH was an *ex vivo* approach, using recombinant retroviruses carrying the LDL receptor gene to transduce hepatocytes, initially in animal models (13–15), then in humans (16, 17). Treated patients showed long-term improvement of lipid profile without signs of toxicity. An *in vivo* approach, employing adenoviral vectors to transfer the LDL receptor gene, has been effective in lowering plasma cholesterol levels in mouse (18) and rabbit models (19, 20) of FH, even though expression of LDL receptor was transient, partly because of the host immune response. This problem could be in part circumvented by the use of DNA–protein complexes capable of targeting the delivery of the LDL receptor gene to hepatocytes (21) or by the use of recombinant adenoviruses containing the very low density lipoprotein receptor gene, which led to a more prolonged metabolic correction (22). To improve the efficiency of *in vivo* retrovirus-mediated gene transfer to the liver, partial liver resection and stimulation of hepatocytes proliferation (by cytotoxic thymidine kinase/ganciclovir (GCV) treatment) were combined with repeated injections of retroviral vectors, carrying

the LDL receptor gene, into the portal vein of Watanabe LDL receptor-deficient rabbits (23). Amelioration of lipoprotein profile in Watanabe rabbits was obtained also by adenovirus-mediated liver expression of *apobec-1*, the catalytic subunit of the enzyme complex which is responsible for the synthesis of apolipoprotein B48 (apoB48) mRNA following post-transcriptional editing of the apoB100 mRNA (24). In rabbits, as well as in humans, the liver normally produces only apoB100 mRNA, being unable to edit endogenous hepatic apoB mRNA.

GH deficiency

GH deficiency is another candidate disease for gene therapy, since conventional treatment with GH requires frequent hormone administrations and shows decreased effectiveness with time. The dwarf little (*lit/lit*) mouse is a model for the human hereditary disorder of isolated GH deficiency type I. This animal model has reduced serum levels of GH and insulin-like growth factor-I (IGF-I), due to an inactivating mutation of the GH-releasing factor receptor. One of the first approaches for correcting the growth disorder in this mouse model was based on the injection of a metallothionein–rat GH fusion gene into eggs. Transgenic animals changed their phenotype to giants. Male animals showed improved fertility, whereas female fertility worsened (25).

As an approach for gene therapy of GH deficiency, genetically engineered myoblasts have been successfully used for GH delivery. The first attempt employed myoblasts transduced by retroviral vectors encoding human GH (26). After injection of transduced myoblasts into the murine muscle, human GH could be detected in serum for up to 3 months (26). Implantation of microencapsulated allogenic myoblasts engineered to secrete murine GH into Snell dwarf mice increased linear growth and body weight of treated animals (27). The allogenic myoblasts, which were protected from immune rejection by enclosure in microcapsules, remained functional for at least 6 months (27). Surprisingly, when these encapsulated myoblasts were implanted into normal mice, the treated animals became significantly shorter, lost weight and showed reduced skeletal growth (28). Bioartificial muscles, i.e. tissue-engineered myoblasts organised into organ-like structures, have been developed in order to ameliorate *in vivo* survival and fusion efficiency of transduced myoblasts (29). Subcutaneous bioartificial muscles secreting recombinant human GH were able to attenuate host skeletal muscle atrophy in a mouse model in which daily GH injections appeared to be ineffective (30). Primary human skeletal muscle cells isolated from fetuses, normal adults and elderly patients with heart failure could be expanded, genetically modified, and tissue-engineered into implantable bioartificial muscles secreting high levels of GH, similar to the murine skeletal myoblasts (31).

Muscle-specific enhancers/promoters have been used to produce biologically active GH or GH-releasing hormone in differentiated muscle cells (32–34). Even though muscle transcription elements can restrict gene expression to muscle fibres (35) continuous GH secretion can cause organ hyperplasia (36, 37). Ideally, a gene therapy strategy for GH deficiency should mimic the physiological pulsatility of GH secretion. A controlled release of GH from skeletal muscle cells has been achieved following implantation of human fibrosarcoma cells into nude mice. These cells expressed two critical domains of a transcription factor as separate polypeptides that interact only in the presence of rapamycin. In cell culture, and *in vivo* upon transplantation of engineered cells into mice, expression of the therapeutic gene was inducible in a rapamycin dose-dependent manner (38). This system allowed long-term regulation of human GH expression when introduced into mice by i.m. injection of two separate adenoviral or AAV vectors, one encoding an inducible human GH gene, the other a bipartite rapamycin-regulated transcription factor (39).

Besides myoblasts, other cell types can be used as GH delivery tools. In this respect, rabbit fibroblasts transfected with human GH cDNA were expanded *in vitro* and implanted under the renal capsule of nude mice, leading to stable delivery of GH for the life-time of the experimental animals (40). A retroviral vector expressing the porcine GH cDNA was used to infect primary rat embryo fibroblasts. Transduced fibroblasts were injected into the peritoneum of syngeneic hypophysectomised rats (41). Implanted cells could secrete biologically active GH *in vivo*, leading to significant skeletal growth up to 57 days post-implantation (41). Microencapsulation and other membrane encapsulation systems can be used to develop non-autologous transduced fibroblast cells for GH delivery (42, 43). The use of keratinocytes for *ex vivo* gene therapy appears to be appealing since these cells can be easily propagated in culture and transferred into human skin. Human GH under the control of human keratin 14 promoter was efficiently produced, secreted, and released into the bloodstream of mice, at levels similar to the circulating GH concentrations in children (44).

Autologous bone marrow stromal cells represent an alternative substrate for *ex vivo* GH-based gene therapy (45). As for target tissues, the liver is an ideal metabolic factory that is also the natural target of adenoviral vectors. An adenoviral vector expressing rat GH cDNA was used to induce constitutive GH expression in hepatocytes of GH-deficient *lit/lit* mice after systemic delivery (36). Treated animals exhibited elevated serum GH values, with restored serum IGF-I concentrations, weight gain, increase in length and normalisation of body composition. Similar results were obtained following i.m. or i.v. injection, or instillation into the duct of the salivary glands of an adenoviral vector

encoding the murine GH gene in GH-deficient Snell dwarf mice (46).

Hypothalamic diabetes insipidus

Hypothalamic diabetes insipidus may be caused by mutations in the arginine vasopressin (AVP) gene. AVP, the anti-diuretic hormone, is synthesised mainly within the supraoptic and paraventricular nuclei of the hypothalamus. After assessing the feasibility of adenovirus-mediated transgene expression in the hypothalamus (47), Geddes *et al.* (48) developed a gene therapy approach for hypothalamic diabetes insipidus. They demonstrated that the stereotactical injection of an adenoviral vector encoding the AVP cDNA into the supraoptic nucleus of the Brattleboro rat, an AVP-deficient animal model of diabetes insipidus, resulted in long-term expression of the gene in magnocellular neurons (48). AVP production was accompanied by reduced daily water intake and urine volume, as well as by increased urine osmolality lasting for up to 4 months. Transient expression of AVP in magnocellular neurons was also demonstrated following injection of naked AVP mRNA into the hypothalamus of Brattleboro rats (49).

CAH

CAH is a group of diseases resulting from reduction or lack of activity of one of the enzymes that regulate cortisol synthesis in the adrenal cortex. Patients with CAH due to 21-hydroxylase deficiency, the most commonly diagnosed enzyme deficiency, cannot adequately synthesise cortisol and mineralocorticoids, leading to an excessive production of adrenocorticotrophin (ACTH) and adrenal androgens. Current treatment is based on steroid replacement, but problems relative to dose adjustment and patient compliance require the development of new therapeutic strategies. The disease is caused by mutations or deletions of the cytochrome P450 21-hydroxylase gene (CYP21). The feasibility of gene therapy for CAH was evaluated in a mouse model with deletion of the CYP21 gene (50). An adenoviral vector carrying the human CYP21 gene under the control of the cytomegalovirus promoter was injected into the adrenal glands of 21-hydroxylase-deficient mice. The highest expression of CYP21 mRNA was observed 2–7 days after injection, followed by a gradual decline. 21-Hydroxylase activity and corticosterone production also reached levels similar to those found in normal mice. Adrenal morphological abnormalities of 21-hydroxylase-deficient mice, i.e. lack of normal zonation and ultrastructural abnormalities of the mitochondria, were reduced 7 days after genetic treatment. A single intra-adrenal injection of the adenoviral construct did not elicit any immune response, suggesting that the *in situ* production of high

levels of steroids has a protective effect, preventing induction of inflammatory responses (50).

Gene therapy for multifactorial endocrine diseases

Although monogenic diseases were the initial focus of gene therapy, more recently multifactorial diseases, such as diabetes mellitus, cancer and cardiovascular disorders have been considered suitable for genetic intervention.

In this regard, cancer has become one of the most important targets of gene therapy. This development occurred not only because of the poor response shown by many tumours to conventional treatments, but also because of the modern view of cancer as a genetic disease at the somatic level, which implies a deeper understanding of the gene-related mechanisms of cell growth and oncogenesis. The role of the host immune system and the role of apoptosis in controlling tumour progression have been exploited to develop strategies that involve augmentation of immunotherapeutic and chemotherapeutic approaches.

Similarly, the comprehension of the role of genetic mechanisms in multifactorial disorders has favoured the concept that genetic modification might be crucial for the design of new treatments. The pathogenesis of the most common diseases is characterised by an aetiological heterogeneity with the involvement of several genes, besides environmental factors. Such a complexity may hamper the development of genetic approaches to therapy as it was originally conceived for monogenic disorders. This is particularly true for some endocrine diseases, such as diabetes mellitus, which require restoration of the exquisite control of gene expression and response to signals of the pancreatic β -cells. The fine regulation of gene expression will also be essential in many other endocrine disorders which require the difficult task of introducing appropriate regulatory signals together with the missing gene.

Diabetes mellitus

Notwithstanding current protocols with diet therapy, insulin supplementation and oral anti-hyperglycaemic drugs, the sharp control of glycaemia, as well as of the long-term complications of the disease, is still problematic. Indeed, diabetes mellitus may be a candidate for a genetic treatment. Potential strategies for gene therapy of diabetes mellitus include: (i) prevention of the autoimmune destruction of β -cells, and thus of the onset of diabetes; (ii) correction of insulin deficiency; and (iii) prevention of end-organ damages (51).

Prevention of β -cell autoimmune destruction can be achieved by genetic modification of either the β -cells or the immune cells (extensively reviewed in (52)).

A strategy to prevent islet damage by induction of tolerance was assessed in animal models of type I diabetes, which received intrathymic injection of islet extracts (53–55) or expressed a proinsulin transgene in the thymus in association with MHC class II molecules (56).

β -Cell impairment and destruction in autoimmune diabetes is mediated in part by interleukin (IL)-1 β , produced by immune cells. Gene transfer of the IL-1 receptor antagonist protein to cultured human islets prevented IL-1 β -induced impairment of the response to a glucose challenge, as well as Fas-triggered apoptosis and nitric oxide production (57). Alternative approaches envisage a reduction in the expression of major histocompatibility molecules (58) or the inhibition of co-stimulatory molecules, such as B7 or its ligand CD28. Indeed, prolonged survival of transplanted β -cells was observed upon engineering β -cells to express the fusion protein CTLA4-Ig, which contains cytotoxic T-lymphocyte-associated protein 4 and an IgG1 Fc portion, in order to block CD28–B7 interaction (59).

β -Cells have also been engineered to secrete molecules that down-modulate the immune response, including IL-4 (60–63), IL-10 (64, 65), transforming growth factor (TGF)- β 1 (65), the p40 subunit of IL-12 (66), anti-CD40 ligands (67) and the E3 region of adenoviruses (67). Inhibition of cytokines that mediate the inflammatory response, such as interferon (IFN)- γ and IL-12, may be a useful tool to treat autoimmune diabetes. To this purpose, i.m. injection of a non-viral vector encoding a soluble IFN- γ receptor/IgG1 heavy chain fusion protein was shown to neutralise IFN- γ and to prevent the onset of diabetes in experimental models (68).

The transfer of genes encoding for molecules that interfere with the cytotoxic activity of activated T-cells or with the apoptotic cascade occurring in β -cells has been explored as a therapeutic strategy for type I diabetes mellitus. FasL-expressing myoblasts co-transplanted with islets into allogeneic murine hosts significantly prolonged islet survival by eliciting the death of activated T-cells that were highly expressing the Fas receptor (69). At variance, FasL-expressing mouse β -cells were not protected in transgenic animals and in allografts (70, 71), probably due to high susceptibility of pancreatic β -cells to Fas-mediated death (70, 72). Better results were obtained with β -cells expressing soluble human FasL (73).

Transduction of β -cells with the *bcl2* gene protected them from apoptosis induced by pro-apoptotic cytokines *in vitro* (74). Similarly, A20 gene transfer to rat islets protected against cytokine-induced apoptosis and cytokine-induced nitric oxide generation (75).

Once β -cell loss has occurred, gene therapy may be employed to restore β -cell function, either by promoting β -cell regeneration or by assisting β -cell replacement. Endocrine pancreatic precursor cells have been

demonstrated to persist even in overt diabetes (76). Generation of β -cells from these precursors has been attempted by expression of a number of differentiation factors, including the vascular endothelial growth factor (VEGF) (77), the islet neogenesis-associated protein (78), the hepatocyte growth factor (79) or members of the *reg* gene family (80). Better results can be achieved when these factors are used in combination with genes that protect from autoimmune attacks or apoptosis.

Replacement of β -cells by transplantation of primary human islet cells has proved disappointing because of the difficulty in obtaining enough islets from cadaveric sources. Genetic modification of cells, either β -cells or other cell types, may provide an efficient source of insulin. There have been many attempts to introduce components of the insulin secretory machinery into non- β -cells to render them suitable substitutes for β -cells. A potential advantage of using autologous non- β -cells is the possibility of evading immune destruction in patients with type I diabetes. To successfully mimic β -cell function, this approach should provide proinsulin synthesis and processing, along with storage and regulated secretion of mature insulin.

Constitutive proinsulin synthesis has been achieved in a variety of cells, including fibroblasts (81), muscle cells (82), hepatocytes (83–85), pituitary cells (86) and cells of exocrine glands of the gastrointestinal tract (87) both *ex vivo* and *in vivo*. Insulin expression could also be activated in non- β -cells from the endogenous locus as shown by introduction of the gene encoding the transcription factor PDX-1 into a glucagonoma cell line, which resulted in activation of the insulin gene, as well as of other β -cell genes (88). Correct proinsulin processing can be obtained in non- β -cells by the introduction of cleavage sites for ubiquitously expressed proteases, such as furin (89) or, conversely, by expression of the β -cell PC2 and PC3 endoproteases in non- β -cells (90, 91).

The reconstitution of regulated insulin secretion in non- β -cells is a more difficult task. In this regard, glucose responsive promoters (i.e. pyruvate kinase and phosphoenolpyruvate carboxykinase promoters) may be employed to regulate the insulin gene transcription (92). However, these promoters respond to insulin, besides glucose, and, therefore, may be less effective in diabetic people. Moreover, the kinetics of feedback loops based on transcriptional changes are much slower than that of the secretory response, with consequent problems of hypoglycaemia and hyperglycaemia.

An alternative to glucose-regulated insulin gene transcription is insulin-regulated release. Neuroendocrine cells, such as the mouse corticotrophic cell line AtT-20, have been engineered for diabetes mellitus gene therapy approaches, exploiting their secretory apparatus, which includes secretory granules and PC2 and PC3 endoproteases (86). Introduction of the glucose transporter GLUT2 gene and of the glucokinase

gene into these cells led to glucose-responsive insulin secretion (93).

The difficulty in reconstituting insulin storage and regulated secretion in non- β -cells makes exogenous β -cells the most realistic candidate cell type for replacing defective β -cell function. Allogeneic and xenogeneic cells have been proposed for β -cell replacement strategies. Xenogeneic β -cells have the advantage of being available in potentially unlimited amounts, but they present problems related to graft rejection and to the presence of xenobiotic viruses (94). In this regard, genetically modified pigs have been produced to overcome xenograft hyperacute rejection (95). To obtain an unlimited source of human pancreatic β -cells, several cell lines from the human fetal and adult pancreas have been developed by expression of dominant oncogenes, but the process of oncogenic transformation leads to dedifferentiation and raises the possibility that unregulated growth might occur in recipients (96). Conditionally transformed β -cells, in which the expression of the oncogene is under the control of an inducible promoter (97) may be safer, even though the risk of changes during prolonged culture of the cell line remains a problem. Engineering human islet stem cells seems a more reasonable approach to overcome this hurdle (98, 99). However, dedifferentiation and loss of insulin synthesis and regulated secretion, due to decreased GLUT2 and glucokinase levels, have been observed also with human islet cells after expansion *in vitro* (100). Improvements in the ability to culture pancreatic cells *in vitro* and to maintain the differentiated state might provide the solution to the problem of developing an unlimited source of β -cells for transplantation. Interesting results have been recently obtained with a human cell line developed from Langerhans islets isolated from a patient with a hyperinsulinaemic hypoglycaemia persisting from infancy (101), a neonatal disease characterised by dysregulation of insulin secretion. Engineering of this cell line by transfection with cDNAs encoding the K_{ATP} channel and PDX1 repaired the genetic defects. A glucose-responsive human insulin-secreting cell line was thus obtained, which could be employed for treating the disease and for generating β -cell lines for cell therapy of diabetes mellitus (101).

Obesity

Recent understanding of the molecular bases of obesity has allowed design of gene therapy approaches for this disease. Leptin, which is exclusively expressed in adipose tissue, is one of the factors that control satiety, as was demonstrated by the obese (ob/ob) mouse model, which lacks plasma leptin due to a nonsense mutation of the gene (102). Daily administration of recombinant leptin to ob/ob mice was shown to induce weight reduction, appetite suppression, and decreased insulin and glucose blood levels (102–104). In the same

animal model, a single injection of adenoviral vectors encoding the murine or the human leptin gene was more effective in accelerating weight reduction than a daily injection of recombinant protein. This result suggests that leptin effects are more prominent at the low steady-state levels which can be achieved by gene therapy (105–108). Hyperleptinaemia modified the phenotype of adipocytes, with reduction of fat deposits, down-regulation of lipogenic enzymes and up-regulation of enzymes for fatty acid oxidation (109). The use of helper-dependent adenoviral vectors, in which the sequences coding for the viral protein are completely eliminated, allowed a significant improvement in safety and resulted in an efficient and long-term *in vivo* delivery of the leptin gene (110). Recombinant AAV vectors may allow long-term expression of therapeutic genes in quiescent cells, such as muscle cells. A single i.m. injection of a recombinant AAV vector encoding mouse leptin in ob/ob mice led to long-term normalisation of metabolic abnormalities, including hyperglycaemia, insulin resistance, impaired glucose tolerance and lethargy (111).

Autoimmune diseases

Endocrine glands are often targets of autoimmune diseases, resulting in either hormone hyperproduction or hypoproduction, which require adequate correction.

Non-specific immunosuppressive regimens, employed for some autoimmune disorders, are complicated by inherent side-effects, including an increased risk of infections and malignancies. Hence, the ideal therapeutic approach should be able to 'silence' only the inappropriate immune response.

Many autoimmune diseases appear to be mediated by the T helper 1 (Th1) subset of CD4+ cells, which express the pro-inflammatory cytokines IFN- γ , IL-2 and tumour necrosis factor- α . By contrast, regulation of autoimmunity appears to be mediated by Th2 cells, through the synthesis of the anti-inflammatory cytokines IL-4 and IL-10. Correcting the imbalance in the activities of Th1 and Th2 lymphocytes can be envisaged as the most appropriate therapeutic approach for some autoimmune disorders (112).

Gene therapy has already been proposed in several animal models of autoimmune diseases to modify the disease course or just to evaluate the inherent treatment toxicity. The first gene therapy trial for an autoimmune disease was started in 1997 in patients affected by rheumatoid arthritis after positive results had been demonstrated in animal models. The pilot study involved *ex vivo* retroviral infection of synovio-cytes with a gene encoding the IL-1 receptor antagonist (IL-1Ra), which blocks IL-1-induced inflammation. Once the patient's synovio-cytes were transduced and tested for absence of infectious contaminants, they were injected intra-articularly. The primary goal of the study was to assess safety, and, after analysis of

IL-1Ra expression, patients underwent joint replacement (113).

One of the great advantages of gene therapy for autoimmune diseases is the ability to deliver disease-modifying proteins locally, avoiding systemic effects. Clinical trials employing the systemic administration of cytokines, antibodies and anti-receptor molecules, have produced clinical benefit (114, 115). However, such an approach can be complicated by emerging side-effects, among which is generalised immunosuppression. In order to bypass this problem adoption of gene therapy in animal models has sought for effectiveness of the local secretion of 'Th2-like' anti-inflammatory cytokines, such as IL-4 and IL-10, of regulatory cytokines like TGF- β , or of receptor antagonists like IL-1Ra. The antigen specificity and migratory properties of memory T-cells allow them to serve as an endogenous system for delivering therapeutic transgenes to autoimmune lesions. Such an approach has already been used to treat the animal model of multiple sclerosis, i.e. the murine experimental allergic encephalomyelitis (116, 117).

Fas–FasL interaction is required for the maintenance of the immune homeostasis. A failure in this interaction may lead to autoimmune and lymphoproliferative disorders (118, 119). Autoreactive T-cells can be eliminated by transferring the gene encoding FasL. Transgenic mice with the FasL gene under control of the insulin promoter have been developed to give protection from insulin-dependent diabetes mellitus (70, 71). However, as mentioned above, lack of protection was observed, probably due to high susceptibility of pancreatic β -cells to Fas-mediated death (70, 72). On the contrary, mice with experimental autoimmune thyroiditis (a murine model of Hashimoto's thyroiditis, characterised by autoreactive T- and B-cell responses and marked lymphocytic infiltration of the gland) showed regression of the disease upon treatment with plasmid DNA coding for FasL under the control of a thyroid-specific promoter (120). Expression of FasL inhibited development of lymphocytic infiltration of the thyroid and induced apoptosis of infiltrating T-cells with total abrogation of the thyroglobulin (TG)-specific cytotoxic T-cell response. The role of gene therapy with FasL for autoimmune thyroiditis in humans remains to be elucidated, as Fas–FasL seems to play a pathogenic role in Hashimoto's thyroiditis (121). A non-viral vector delivery of the IL-10 gene has also been employed in an attempt to treat mice affected by experimental autoimmune thyroiditis (122). The rationale for this approach was given by the curative effects demonstrated by i.v. administration of IL-10 in this animal model (123).

The future goal for gene therapy of endocrine autoimmune disorders is the identification of the best gene, or combination of genes, to use in each disease and of the best system to obtain a long-lasting response.

Endocrine tumours

Current approaches for cancer gene therapy (124) can be divided into five categories (Table 4): transfer of genes with direct anti-tumour effects (including antisense oligonucleotides or ribozymes to inhibit oncogenes, or introduction of tumour suppressor genes or genes with anti-angiogenic effects); transfer of genes that activate drugs to their toxic form (suicide genes); transfer of genes that enhance the immune response against cancer (either by genetic modification of tumour cells or by immune effector cells); and transfer of genes which decrease toxicity from chemotherapy, such as the multidrug resistance gene *MDR1*.

Corrective cancer gene therapy As for most cancers, endocrine tumours result from a multistep process, including loss of cell cycle control, neo-angiogenesis and evasion of immune control. Rarely, endocrine tumours occur as part of an inherited cancer syndrome, such as multiple endocrine neoplasia and the Li–Fraumeni syndrome. Most molecular abnormalities consist of the loss of putative tumour suppressor genes or of the activation of proto-oncogenes. Tumour suppressor genes regulate gene transcription and cell proliferation, and inactivation of both copies of the gene is needed to abrogate the anti-proliferative function. Thus, supplementation of a correct copy of the tumour suppressor gene in cells with homozygous loss of function could restore normal growth, induce tumour cell dormancy or apoptosis. At variance, mutation of only a single copy of a proto-oncogene is required for malignant transformation of a cell. Antisense oligonucleotides, ribozymes, and intracellular single-chain antibodies can be used to reduce the expression of oncogenes.

Gene therapy of cancer, in its most direct form, should aim at replacing a mutated gene with its correct form, or at suppressing the abnormal oncogenic function. At present, however, such a corrective gene therapy faces the insurmountable task that gene replacement, or gene suppression, should simultaneously involve a number of different genes, and should be applied to all tumour cells to reverse the malignant phenotype. Hence, corrective gene therapy

seems to be quite difficult to propose as a single therapeutic approach.

Antisense gene therapy by the use of antisense oligonucleotides is aimed at suppressing the expression of a harmful gene, for example a proto-oncogene or a viral gene. Antisense oligonucleotides are short stretches of DNA (about 20 nucleotides in length) or modified DNA that contain a base sequence complementary to a target RNA. By annealing to the target RNA, they interfere with its transport, splicing and translation. In some cases, the hybrid structure between antisense oligonucleotides and target molecules forms a substrate for cellular RNases. Structural and chemical modifications of oligonucleotides (phosphorothioate analogues being the most commonly used) have been developed to enhance affinity and exonuclease resistance, and to reduce toxicity. Non-viral and viral vector systems have been employed to favour oligonucleotides delivery and/or intracellular production. Besides specific antisense effects, oligonucleotides may work via non-antisense mechanisms by binding to growth factors or to their receptors, thus interfering with cellular proliferation, an activity that can be exploited in cancer gene therapy.

Inhibition of target genes can be achieved also by ribozymes. Ribozymes are catalytic RNA molecules that recognise their target RNA in a sequence-specific manner and cleave the RNA strand. Ribozymes may be used as therapeutic tools because of their ability either to inhibit deleterious gene expression (oncogenes, viral transcripts) by cleavage of the target mRNA or even to repair mutant cellular mRNAs (125, 126). There are several classes of ribozymes, with either *cis*-cleaving or *trans*-cleaving activity, among which are small pathogenic RNAs. These ribozymes contain different catalytic motifs (hammerhead, hairpin, axehead) and flanking arms capable of base-pairing with the substrate. Since the arms of ribozymes that base-pair to substrate RNA are functionally separable from the moieties that effect cleavage, the substrate specificity of ribozymes can be altered, within certain constraints, to allow *trans*-cleavage of target transcripts. Many of the problems raised by the employment of ribozymes are similar to those outlined for antisense oligonucleotides, i.e. efficient entry into the cell, stability and specificity of substrate targeting.

Some endocrine-related tumours, such as breast and prostate cancer, have been targeted using antisense oligonucleotides or ribozymes. IGFs are important mitogens for a variety of tumour cells, including endocrine cancers and endocrine-related cancers (127). An anti-IGF-I receptor antisense strategy has been successfully used against breast cancer cell lines (128). Similarly, cleavage of IGF-II mRNA by a hammerhead ribozyme reduced prostate cancer cells growth (129). Hammerhead ribozymes have also been designed to specifically cleave the androgen receptor mRNA (130) and the oestrogen receptor mRNA (131), which play key roles

Table 4 Cancer gene therapy strategies.

1. Corrective cancer gene therapy
 - a. Inactivation of oncogenes by antisense and ribozymes
 - b. Introduction of tumour suppressor genes
2. Anti-angiogenic gene therapy
3. Suicide gene therapy
4. Immunomodulating gene therapy
 - a. Active immunisation (modification of tumour cells to enhance immunogenicity or immunisation with genes encoding tumour antigens)
 - b. Genetic modification of immune effector cells
5. Drug resistance genes to decrease toxicity from chemotherapy

in prostate and breast cancer growth respectively. Transient transfection of prostate cancer cells with the androgen receptor ribozyme led to decreased activity and expression of androgen receptors, without deleterious side-effects (130). Human MCF-7 breast cancer cells proliferation and entry into the S phase of the cell cycle decreased upon treatment with the oestrogen receptor-specific ribozyme (131).

While oncogene alterations are usually dominant, tumour suppressor genes become tumorigenic through loss of function and tend to act in a recessive manner. *p53* is the most commonly mutated tumour suppressor gene in human cancer and seems to play a prominent role in some endocrine tumours (132, 133). Wild-type *p53* protein is involved in G1 cell cycle arrest and apoptosis of DNA-damaged cells, and is therefore crucial in preventing mutation or deletion in functional genes. Restoration of wild-type *p53* expression by the introduction of the wild-type *p53* gene has recently been used in several cancer models and in human clinical trials of anti-cancer gene therapy (134). Introduction of wild-type *p53* in neoplastic cells can significantly inhibit growth and angiogenesis or can induce apoptosis in a variety of *p53* mutant cancer cells *in vitro*, including thyroid carcinomas (135–137). Since the presence of functional *p53* has been shown to modulate chemoresistance, another possible advantage of the restoration of wild-type *p53* may be sensitisation to chemotherapy and radiotherapy. Indeed, the combination of *p53* gene transduction with radiation or chemotherapy resulted in an enhanced cytotoxic effect on thyroid carcinoma cell lines (137, 138), even though the effectiveness of *p53*-based gene therapy was limited to cells carrying an inactive *p53* (139).

The cell cycle regulator genes provide an additional target for corrective gene therapy. Among these, the p105Rb product of the retinoblastoma tumour suppressor gene (*Rb*) is one of the most critical regulators of cellular proliferation. The Rb protein, when unphosphorylated, is responsible for the cell cycle arrest by inhibition of the activity of the E2F family of transcription factors. Normal cell cycle progression requires inactivation of Rb through phosphorylation by cyclin-dependent kinases. In humans, Rb is inactivated in all familial and sporadic retinoblastomas, in 90% of small cell lung carcinomas, and in a variety of other tumours. Mice with a single wild-type copy of *Rb* (*Rb*^{+/−}) develop *Rb*-deficient melanotroph tumours of the pituitary intermediate lobe, and, in some cases, C-cell thyroid carcinomas and hyperplasia of the adrenal medulla (140, 141). To evaluate the tumour suppressive potential of the wild-type *Rb* gene *in vivo*, a recombinant adenoviral vector expressing human *Rb* cDNA was delivered by direct injection into the pituitary intermediate lobe (142). Intratumoural *Rb* transfer decreased tumour cell proliferation, re-established innervation by growth-regulatory dopaminergic neurons, and significantly prolonged the life spans of

treated animals compared with controls. Expression of a human *Rb* transgene in the *Rb*^{+/−} mice suppressed tumour progression in a variety of tissues (143). Moreover, the frequency of lung metastases in *Rb*^{+/−} mice was reduced by repeated i.v. administration of *Rb* cDNA by non-viral vectors.

Restoration of other tumour suppressor genes expression, such as p16 and p27, may be employed to inhibit growth and to suppress tumorigenicity of endocrine neoplasms (144–147). Indeed, stable transfection of p16 in the mouse pituitary cell line AtT20, which carries a biallelic deletion of the p16 locus, arrested cells in the G1 phase and reduced cell proliferation (148).

Anti-angiogenic gene therapy Tumour growth and metastasis are dependent upon the ability of cancer cells to establish a blood supply through the process known as neoangiogenesis. This complex process is regulated by numerous factors, some being endowed with angiogenic properties, i.e. VEGF, platelet-derived growth factor- α , basic fibroblast growth factor (bFGF), epidermal growth factor and IL-8, while others act as endogenous inhibitors of angiogenesis, i.e. thrombospondin-1, platelet factor 4 (PF4), angiostatin and endostatin.

Anti-angiogenic therapies so far devised target different steps of the anti-angiogenic process, by inhibiting expression of angiogenic molecules, by overexpressing anti-angiogenic molecules, or by targeting tumour endothelial cells via endogenous angiogenic inhibitors or artificially constructed targeting ligands. Destruction of tumour vasculature can lead to tumour regression due to apoptosis (149). Gene therapy has been advocated as a means to deliver anti-angiogenic factors to the tumour vasculature. It offers the advantage of high local production of factors with low systemic toxicity. Since the first report of anti-angiogenic gene therapy with a dominant negative mutant of the VEGF receptor (150), new important tools to suppress tumour growth and spread have been developed, which could be employed also for endocrine neoplasms. The main attempts are directed at: (i) suppressing expression of angiogenic factors with antisense oligonucleotides or ribozymes; and (ii) transferring genes that disrupt receptors for angiogenic factors (such as dominant negative functioning extracellular domains of the VEGF receptor flt-1) or encode anti-angiogenic molecules, i.e. thrombospondin-1, PF4, angiostatin and endostatin (151). Although many reports have established the ability of anti-angiogenic gene therapy to inhibit tumour growth and metastatic spread, this therapeutic approach often results in incomplete suppression of tumour growth and metastasis, and no tumour regression (151). This problem may be circumvented in part with a combined gene therapy approach, employing different angiogenesis inhibitors together with cytokine or suicide genes.

Angiogenesis has been demonstrated to play an important role in tumour cell proliferation and in hormonal secretion of endocrine tumours. Thyroid neoplasms show an increased expression of angiogenic factors, including VEGFs (152–154) and angiopoietin-2, as well as their tyrosine kinase receptors (153). In particular, overexpression of angiopoietin-2 and VEGF is associated with thyroid tumour progression from a prevascular to a vascular phase. Furthermore, thyroid cancers with lymph node spread show increased expression of VEGF-C, whereas aggressive tumours capable of haematogenous spread show a decreased production of thrombospondin-1 (153). A higher number of microvessels have been found in papillary thyroid carcinomas with distant recurrences. This finding, even if controversial, has been correlated with disease-free survival (153, 155, 156). A correlation between microvessel count and prognosis has been suggested also for medullary thyroid carcinomas (156). To investigate the role of VEGF in the angiogenetic process associated with the development of thyroid carcinomas, VEGF was stably transfected into a poorly tumorigenic cell line which expressed low basal levels of VEGF. As compared with parental cells, VEGF-overexpressing cells formed well-vascularised tumours when injected s.c. into nude mice (157). Moreover, antisense suppression of VEGF expression in a highly tumorigenic anaplastic cell line led to the development of small and poorly vascularised tumours *in vivo* (157). The feasibility of anti-angiogenic therapy to control the growth of anaplastic thyroid carcinomas has been demonstrated also with an inhibitor of angiogenesis in an animal model (158).

Other endocrine tumours may become target of anti-angiogenic therapy. Indeed, angiogenesis plays an important role in the development of the adrenal cortex (159, 160) and in adrenocortical tumour cell proliferation (161). Adrenocortical carcinomas seem to be associated with increased endothelial cell proliferation (161), whereas malignant pheochromocytomas and paragangliomas show an increased number of intratumoural microvessels (162).

The normal anterior pituitary is a well-vascularised tissue, whereas pituitary tumours are less vascular than normal pituitary tissue, suggesting that angiogenesis may be inhibited in these tumours (163, 164). However, although these tumours are less vascular overall, they may have induced new vessel development from the systemic circulation, altering oxygen delivery and escaping hypothalamic influences on hormone production (165–167). A series of vasogenic factors are expressed and produced within the pituitary gland, including some factors involved in angiogenesis (168). Pituitary folliculostellate cells can produce both anti-angiogenic factors and angiogenic factors, including VEGF (169) and bFGF (170). These cytokines act in an autocrine/paracrine manner, and are regulated by hypothalamic factors, such as pituitary adenylate

cyclase-activating polypeptide and IL-6 (171). Oestrogen-induced rat pituitary lactotroph tumours and rat pituitary GH/prolactin (PRL)-secreting GH3 tumour cell line overexpress VEGF and its co-receptor neuropilin-1 (172), suggesting a role in the modulation of pituitary tumour angiogenesis. Development of oestrogen-induced rat prolactinomas is also associated with increased expression of pituitary tumour transforming gene (PTTG) and bFGF (173). A concomitant overexpression of PTTG and bFGF has also been demonstrated in human pituitary tumours (173). As oestrogens and bFGF both induce PTTG, and PTTG expression coincides with the early stages of lactotroph cell transformation (from normal to hypertrophic cell), these factors may play a prominent role in pituitary tumorigenesis, and represent potential targets for therapeutic intervention (173).

Suicide gene therapy Suicide gene therapy is defined as the use of a gene that converts a pro-drug into a toxic substance; independently, the gene product and the pro-drug are non-toxic. The prototype of this approach exploits the requirement for the intracellular phosphorylation of GCV by the herpes simplex virus thymidine kinase gene product (HSV-TK). Activation generates a toxic drug metabolite that inhibits DNA synthesis, inducing cell death. The specificity of the system can be increased by using tumour-specific promoters upstream of the suicide gene so that the gene is not transcribed within normal tissues. An interesting feature of the HSV-TK/GCV system is the 'bystander' killing of non-transduced cells. The mechanisms that are responsible for this effect have not been fully defined, but are likely to include transfer of non-diffusible phosphorylated GCV to neighbouring cells through gap junctions, endocytosis by non-transduced cells of debris containing toxic GCV, and stimulation of host anti-tumour immune responses. A number of other suicide/prodrug systems have been employed. Among these are: *E. coli* cytosine deaminase that converts the relatively non-toxic 5-fluorocytosine to the chemotherapeutic agent 5-fluorouracil; cytochrome P-450 2B1 that catalyses cyclophosphamide conversion and ifosfamide activation; *E. coli* nitroreductase that converts 5-aziridinyl-2,4-dinitrobenzamide to a potent DNA crosslinking agent; and *E. coli* purine nucleoside phosphorylase that generates toxic purine nucleoside analogues (174).

The effectiveness of suicide gene therapy has been explored in a variety of neoplasms, including endocrine tumours. The HSV-TK/GCV scheme was demonstrated to be effective against thyroid and pituitary tumours in cell cultures and in animal models. In this regard, human follicular and anaplastic thyroid carcinoma cell lines, transduced with a retroviral vector containing HSV-TK under the control of the cytomegalovirus promoter, were killed by GCV in a dose- and time-dependent manner. Tumour regression was also

observed in nude mice. A bystander effect and radio-sensitisation were documented *in vitro* and *in vivo* (175). To develop thyroid-specific retroviral vectors the bovine TG promoter was introduced to control suicide gene expression (176). Selective killing of TG-producing cells was observed upon GCV treatment (176). A synthetic tandemly repeated TG promoter seemed to enhance promoter activity (177). Another strategy to improve TG promoter activity exploited the use of the Cre-loxP system (178). To this end, two recombinant adenoviral vectors were used, one with the Cre recombinase gene under the control of the TG promoter, the other with a stuffer sequence, flanked by the loxP sequences and including the neo-resistance gene. The latter gene was placed between the strong chimeric CAG promoter and the HSV-TK gene. In this system, the Cre recombinase is expressed when the TG promoter is active, thus removing the stuffer sequence and inducing HSV-TK expression under the transcriptional control of the CAG promoter. The cytotoxic effect of HSV-TK/GCV obtained *in vitro* with the TG promoter and the Cre-loxP system was 5- to 10-fold higher than that obtained with the TG promoter alone. Enhanced tumour growth inhibition was also observed in *in vivo* tumour models (178).

A tissue-specific approach was attempted also for medullary thyroid carcinomas, exploiting the promoter sequence of the calcitonin gene to drive selective expression of HSV-TK in tumour cells injected *in vivo*, with reduced side-effects (179).

Adenoviral vectors have been demonstrated to be effective and safe delivery vehicles for the pituitary gland (3, 180–185). Adenoviral vectors were employed to transduce pituitary adenoma cell lines and animal models with HSV-TK (181, 186). Cell-type-specific expression of the therapeutic gene was achieved using the promoters of the GH, glycoprotein hormone α -subunit, and PRL genes (181, 187). In addition to cell-type-specific expression of the therapeutic gene, regulation of transgene expression is foreseeable for gene therapy of many endocrine disorders. The tetracycline-inducible system, which allows gene expression to be regulated by the tetracycline analogue doxycycline, has been used to develop a PRL-specific and regulatable adenoviral vector (188, 189). By driving the expression of the tetracycline transactivator through the PRL-specific promoter, expression of the inducible transgene was restricted to both lactotrophic tumour cell lines and PRL-positive cells in primary anterior pituitary cultures (188, 189).

Targeting therapeutic genes to specific cell types is particularly relevant for pituitary adenoma gene therapy in order to spare normal pituitary cells and neighbouring tissues. This aim could be also achieved by retargeting viral vector tropism so that the vectors recognise and bind pituitary-specific surface molecules, such as hypothalamic hormone receptors, which are often overexpressed in pituitary tumours (190, 191).

Recombinant temperature-sensitive HSV type 1 vectors have been employed for gene transfer into tumours and normal anterior pituitary cells (192). However, neurovirulence of this vector system limits its potential use for gene therapy in humans, even though it can be used as a delivery system to generate transient transgenesis for biological studies (193).

Transcriptionally targeted gene therapy is feasible also for other endocrine cancers, exploiting *cis*-acting regulatory sequences of tissue-specific genes. Promoter/enhancer elements of steroidogenic enzymes could be employed to target transgene expression in steroid-secreting cells. A chimeric enhancer/promoter element, containing both the CYP11B1 promoter and the P450SCC enhancer was employed to target HSV-TK expression in adrenocortical carcinoma cells. HSV-TK expression and GCV sensitivity of stably transfected adrenal cancer cells was enhanced by treatment with factors acting through the cAMP pathway, such as ACTH (194).

A limitation for targeted gene therapy approaches employing enhancer/promoter elements of genes expressed by differentiated cells, such as hormones, receptors or enzymes, is the risk of decreased transgene expression in tumour cells due to dedifferentiation. To overcome this problem, regulatory sequences of genes that are highly and specifically expressed in malignant cells, such as IGF-II in adrenocortical carcinomas (195) or oncofetal fibronectin in thyroid carcinomas (196), should be employed.

The iodide-concentrating ability of thyroid cells, which is based on the tissue-specific expression of the sodium/iodide symporter gene (*NIS*), allows for diagnostic radionuclide thyroid imaging with ^{123}I and $^{99\text{m}}\text{Tc}$. Radioiodine therapy of differentiated thyroid carcinomas using ^{131}I can also be pursued, with minimal systemic side-effects, except for the need for replacement therapy. The rat *NIS* has been transferred into cancer cells by a retroviral vector to mimic the iodide uptake of thyroid follicular cells. *NIS*-mediated accumulation of ^{123}I by transduced cancer cells allowed scintigraphic detection of tumours *in vivo*. Selective killing of *NIS*-transduced cells by the induced accumulation of ^{131}I was demonstrated *in vitro* (197). Thus, *NIS*-based gene therapy may have both diagnostic and therapeutic applications for cancer.

Morbidity and mortality of patients with endocrine tumours are often due more to hormone overproduction rather than to metastatic disease. In some cases refractory to conventional surgical and pharmacological treatment, such as Cushing's syndrome, acromegaly and endocrine pancreatic tumours, gene therapy may be an effective approach. In this regard, human lactotroph adenoma cells have been transduced with an adenoviral vector encoding tyrosine hydroxylase, a rate-limiting enzyme in the biosynthesis of dopamine, in an attempt to decrease PRL release (3). A similar approach could be used for other types

of tumours. For example, GH-secreting adenomas could be treated with somatostatin gene transfer.

Immunomodulating gene therapy Immunomodulating gene therapy has been developed in an attempt to stimulate cellular immune responses against tumour-specific antigens. One method is to genetically modify the tumour cell to have it express cytokines (e.g. IL-2, IL-12, granulocyte-macrophage colony-stimulating factor, or IFN- γ) that attract antigen-presenting cells such as dendritic cells and macrophages, and to activate a systemic T-cell immune response. Another method is to genetically modify the tumour cell itself to become a professional antigen-presenting cell through the expression of MHC molecules, co-stimulatory molecules such as B-7, and cytokines genes necessary for T-cell activation and proliferation. To avoid costly and time-consuming processes involved in generating tumour vaccines by *ex vivo* approaches, *in vivo* strategies to enhance immune response have been developed, such as vector-mediated delivery of cytokine genes.

Another immunological approach involves the use of vectors for the expression of tumour-associated antigens (vaccines). Recombinant vaccines may be either viral, bacterial or 'naked' DNA (198). Recombinant viral vaccines offer the advantage of intrinsic immunogenicity, whereas bacterial vaccines have cellular tropism for monocytes and macrophages, and possess enteric routes of infection, providing the possibility of oral delivery (198). 'Naked' DNA vaccines, which can persist and express encoded genes for long periods of time after i.m. injection, have been demonstrated to mediate both antibody-mediated and cellular immune responses against encoded gene products, even if they are less potent than other recombinant vaccines (199).

Another potential method of manipulating the immune response against cancer is to utilise dendritic cells (200), which are highly potent antigen-presenting cells capable of stimulating and activating quiescent lymphocytes. Stable introduction of tumour-antigen genes into dendritic cells may allow presentation of multiple immunogenic epitopes of the same antigen, as well as the constitutive expression of the antigen *in vivo*. In addition, introduction of cytokine genes into dendritic cells may enhance the anti-tumour response.

Genetic immunotherapy showed interesting results in a model of medullary thyroid carcinoma *in vitro* and *in vivo*. *In vitro* infection of murine medullary thyroid carcinoma cells with an adenoviral vector harbouring the mouse IL-2 gene abrogated their tumorigenicity and induced a long-lasting state of immunity in syngeneic BALB/C mice (201). *In vivo*, intratumour injection of the adenoviral vector resulted in the rejection and/or stabilisation of pre-established tumours in treated mice (202) without significant toxicity to other organs (203).

Combined gene therapy approach A new treatment strategy combining two different modalities, enzyme-directed prodrug activation (tumour suicide) along with cytokine-promoted tumour rejection, has been recently devised to amplify the anti-tumour response, and proved to be efficacious in animal models (204). A bicistronic retroviral vector co-expressing HSV-TK and human IL-2 genes has been designed to pursue this new approach of cancer gene therapy in humans (205, 206) and successfully employed in a pilot study to treat four patients with recurrent glioblastoma multiforme (207, 208). A similar construct has been developed to treat human thyroid carcinomas. In this regard, the HSV-TK and human IL-2 genes, separated by an internal ribosome entry site to allow the simultaneous expression of the two genes from the same transcript, were cloned into the MFG retroviral vector. Transcriptionally targeted thyroid-specific vectors were generated replacing the viral enhancer in the long terminal repeat with the enhancer sequence of the human TG gene (209). *In vitro* experiments showed selective killing of transduced thyroid carcinoma cells by GCV treatment, and secretion of biologically active IL-2. Such a combined approach, employing both cytokine and suicide genes, seems to elicit a potentiated anti-cancer effect *in vivo*, as observed in a mouse model of medullary thyroid carcinoma inoculated with both HSV-TK-transduced carcinoma cells and IL-2-transfected cells (210).

Conclusions

Scientific progress in biomedical research has revealed the molecular mechanisms of many human diseases. Although several endocrine disorders are caused by the inheritance of a single, functionally defective gene, the molecular bases of most endocrine diseases relate to acquired genetic defects. These defects would eventually require an aetiological (non-pharmacological) genetic intervention. Present premises seem to favour intervention for endocrine tumours, but many combined approaches of cell and gene therapy are foreseeable also for endocrine and metabolic disorders.

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