

Endocrine Aspects of Cancer Gene Therapy

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The field of cancer gene therapy is in continuous expansion, and technology is quickly moving ahead as far as gene targeting and regulation of gene expression are concerned. This review focuses on the endocrine aspects of gene therapy, including the possibility to exploit hormone and hormone receptor functions for regulating therapeutic gene expression, the use of endocrine-specific genes as new therapeutic tools,

the effects of viral vector delivery and transgene expression on the endocrine system, and the endocrine response to viral vector delivery. Present ethical concerns of gene therapy and the risk of germ cell transduction are also discussed, along with potential lines of innovation to improve cell and gene targeting. (Endocrine Reviews 25: 1–44, 2004)

- I. Introduction
 - A. Efficacy and toxicity of gene therapy
 - B. Gene therapy strategies for targeting endocrine and endocrine-related tumors
- II. How to Exploit the Endocrine System for Regulating Therapeutic Gene Expression
 - A. Physiologically regulated gene expression systems
 - B. Pharmacologically regulated gene expression systems
- III. Endocrine Cell-Specific Genes as New Therapeutic Tools
 - A. Sodium/iodide symporter
 - B. Noradrenaline transporter
 - C. Somatostatin receptor
- IV. Endocrine Side Effects of Gene Therapy
 - A. Nonviral vectors
 - B. Viral vectors
 - C. Ectopic expression of cytokines as therapeutic genes
 - D. Inhibition of hormones and growth factors
- V. Oncolytic Virus Infection of Endocrine Cells
 - A. Oncolytic adenovirus
 - B. Oncolytic herpes simplex viruses
 - C. Reovirus
- VI. Endocrine Response to Viral Vector Delivery
 - A. HSV-based vectors
 - B. Adenoviral vectors
 - C. Newcastle disease virus
- VII. Risk of Germ Cell Transduction and Present Ethical Concerns
 - A. Risk of germ cell transduction
 - B. Ethical issues
- VIII. Summary

I. Introduction

THE CONCEPT OF gene therapy developed from the observation that certain diseases are caused by inheritance of a single functionally defective gene, and, therefore, could be potentially treated by the introduction and expression of a normal copy of the mutant or deleted gene in the host cells. This original concept of gene therapy as “gene supplementation” has rapidly switched to the more general one comprising any strategy that uses genetic material to prevent or cure a variety of diseases, including multifactorial and somatic genetic diseases, such as cancer. Indeed, cancer has become by far the most important indication for gene therapy in clinical trials, representing 68.5% of gene therapy protocols, enrolling a total of 2392 patients worldwide (The Journal of Gene Medicine; www.wiley.co.uk/genmed/). First results from clinical trials, however, indicate that some key issues still need to be addressed before including gene therapy as a standard of care in the management of cancer patients. Critical problems to overcome are low efficiency and lack of selectivity of currently available gene transfer systems. These aspects have become particularly relevant with the application of systemic delivery of nonreplicating, or even replication-competent, viral vectors in cancer gene therapy clinical trials, which sometimes may have unpredictable toxicity. Effects related to therapeutic genes or gene transfer procedures on the endocrine system have rarely been addressed, although the interplay between the endocrine system and the immune-inflammatory response to viral infection or transgene delivery suggests that endocrine cells may play an important role in the acute response to therapeutic gene/vector administration. Moreover, effects of vector delivery on hormone production may be predicted on the

Abbreviations: AAV, Adeno-associated virus; CAR, coxsackie-adenovirus receptor; EcR, ecdysone receptor; EGF, epidermal growth factor; EGFR, EGF receptor; Egr, early growth response; ER, estrogen receptor; GCV, ganciclovir; GM-CSF, granulocyte macrophage-colony stimulating factor; GR, glucocorticoid receptor; HIV-1, HIV type 1; HPA, hypothalamus-pituitary-adrenal; 3β -HSD, 3β -hydroxysteroid dehydrogenase/ $\Delta 5$ - $\Delta 4$ isomerase; HSP, heat-shock protein; HSV, herpes simplex virus; HSV-TK, HSV-thymidine kinase; IFN, interferon; IGF-IR, IGF type I receptor; LH-R, LH receptor; LTR, long terminal repeat; MDR, multidrug resistance; MHC, major histocompatibility complex; Mo-MLV, Moloney murine leukemia virus; NAT, noradrenaline transporter; NIS, sodium/iodide symporter; 4-OHT, 4-hydroxy-tamoxifen; OTC, ornithine transcarbamylase; pfu, plaque-forming unit; PRL, prolactin; PSA, prostate-specific antigen; RAR, retinoic acid receptor; RXR, retinoid X receptor; sst, somatostatin receptor subtype; TG, thyroglobulin; TPO, thyroperoxidase; TR, thyroid hormone receptor; VEGF, vascular EGF; X-SCID, X-linked severe combined immunodeficiency.

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basis of studies on the pathophysiology of viral infection of endocrine cells. Knowledge of the interplay between vector delivery and endocrine response may be useful in the choice and design of vectors with an improved safety profile. Another contribution of endocrinology to the field of gene therapy derives from the growing understanding of the mechanisms underlying regulation of gene expression: it is becoming evident that tight regulation of hormone production and hormone receptor functions may be exploited in the development of regulated and targeted vector systems, not only for applications in endocrine glands, but also for other tissues.

After a brief introduction on efficacy and toxicity of gene therapy in clinical trials for the treatment of cancer and the strategies developed so far to target endocrine and endocrine-related tumors, this review will focus on the endocrine aspects of cancer gene therapy. These include the possibility to exploit molecular mechanisms of regulation of hormone activity to control therapeutic gene expression, the use of endocrine cell-specific genes as therapeutic tools, the potential side effects of cancer gene therapy on the endocrine system, the neuroendocrine response to vector delivery, the effects of ectopic expression of cytokines as therapeutic genes, and side effects related to hormone or growth factor inhibition. The risk of germ line cell transduction and present ethical concerns will be also addressed.

A. Efficacy and toxicity of gene therapy

1. Overview. The concept of cancer gene therapy derives from new understandings of the molecular biology of cancer and the complex interactions between tumor cells and the immune system. This knowledge has been exploited to develop strategies to selectively target tumor cells or to stimulate the immune response against tumor antigens. Current therapeutic approaches and results from clinical trials of cancer gene therapy, which have been reviewed recently (1, 2), are summarized in Fig. 1 and Table 1. It is apparent that, since the development of the first cancer gene therapy clinical trials in the early 1990s, clinical results are still largely unsatisfactory, notwithstanding efforts to improve gene transfer tools and therapeutic genes.

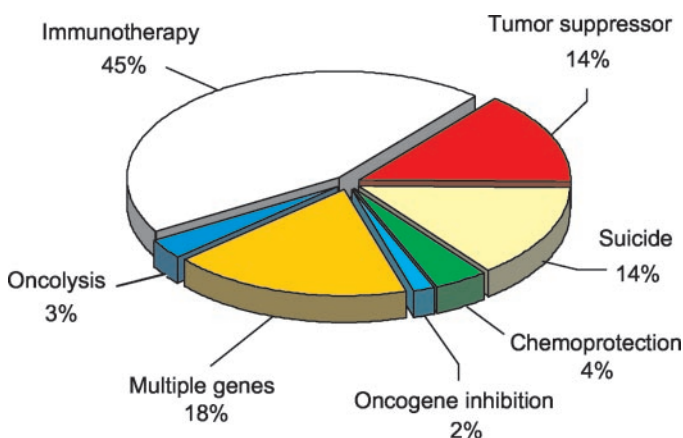


FIG. 1. Clinical trials for cancer gene therapy (n = 403) grouped according to the therapeutic strategy employed (data were obtained from the Journal of Gene Medicine, www.wiley.co.uk/genmed/ and from the published literature).

Strategies and perspectives of clinical trials of gene therapy, including cancer gene therapy, have been impressively affected by two recent events: the death of the first patient due to gene therapy itself and the first clinical successes of gene therapy (3).

The first event was the tragic death, in September 1999, of an 18-yr-old patient with ornithine transcarbamylase (OTC) deficiency enrolled in a clinical trial at the University of Pennsylvania (4). This event focused the attention of the scientific world on the potential risks associated with gene transfer and the need to accurately record and report serious adverse events in gene therapy trials. In the Penn study, patients with partial OTC deficiency were treated via the hepatic artery with escalating doses of a nonreplicating adenovirus carrying the gene encoding for OTC (5). Intravascular administration of the vector resulted, in some patients, in some of the clinical symptoms also reported in other studies using adenoviral vectors, *i.e.*, transient fever, myalgias, elevation in liver enzyme and cytokine levels, reversible hypophosphatemia, thrombocytopenia, and anemia (6). However, in a male patient enrolled in the highest dose cohort who received approximately 4×10^{13} viral particles, the initial mild symptoms progressed to acute respiratory distress syndrome and subsequent multiorgan failure, resulting in the patient's death 4 d after treatment. A report from the National Institutes of Health Recombinant Advisory Committee (7) attributed the death to a systemic, adenovirus vector-induced shock syndrome, due to a cytokine cascade that led to disseminated intravascular coagulation, acute respiratory distress, and multiorgan failure. Moreover, postmortem examination revealed bone marrow red cell aplasia. It was suggested that the high dose of adenoviral vector quickly saturated available receptors for the vector and then spilled to other organ systems, including the bone marrow, thus inducing a systemic immune response. Adenoviral vector capsid proteins likely contributed to the patient's immune response (8, 9).

The positive events were the first conclusive evidence that gene therapy can be successful in humans. These regarded a clinical protocol in patients with hemophilia based on im injection of recombinant adeno-associated viral (AAV) vectors (10) and a gene therapy clinical trial in children with X-linked severe combined immunodeficiency (X-SCID) via retrovirus transduction of hematopoietic stem cells (11, 12).

In the first study, after preclinical evidence of correction of the hemophilic phenotype in animal models (13, 14), efficient transduction and expression of factor IX was achieved in three hemophilia B patients receiving im injection of an AAV vector encoding blood coagulation factor IX. The procedure was safe, and a mild improvement of clinical condition was achieved (10). In the second study, four of five children with X-SCID due to a deficiency of the γ chain, who were treated with autologous bone marrow stem cells and transduced *ex vivo* with the γ gene, showed evidence of long-term correction of the immune deficiency (11, 12). However, the great optimism for the potential clinical benefits of gene transfer generated by these results was frozen by the announcement of serious adverse events observed in the clinical trial of gene therapy for X-SCID, which led the U.S. Food and Drug Administration and the American Society of Gene Therapy to

TABLE 1. Results from gene therapy clinical trials

Gene therapy protocol	Therapeutic gene(s)	Targeted tumor and results
Immunotherapy	IL-3, IL-4, IL-7, IL-12 GM-CSF, TNF- α , TGF- β IFN- α , IFN- β , IFN- γ Antigens (gp100, MART-1, melan-A, muc-1, CEA, PSA, HLA-B7/ β 2-microglobulin) Receptors (B7-1, scFvFc-Zeta TCR)	Partial response in up to 20% of patients treated with IL-2 or IL-7 gene transfer (21, 26, 74). Local response in 18% of patients receiving intratumoral injection of HLA-B7/ β 2-microglobulin DNA-liposome complex for metastatic melanoma (23)
Tumor suppressor gene therapy	<i>p53</i> <i>BRCA1</i>	Partial tumor responses in phase I and pilot studies in lung, head and neck, bladder, and metastatic liver tumors (25, 27, 32, 51, 78, 79, 90, 634–637), but no response in a phase II study in non-small-cell lung carcinoma in combination with chemotherapy (31) Three cases of partial tumor response in phase I trial (28), but no response in a phase II trial for ovarian cancer (80)
Oncogene suppression	<i>p16</i> <i>IGF-IR</i> AS ODN <i>TGF-β2</i> AS ODN <i>c-myc</i> or <i>c-fos</i> AS ODN <i>EGFR</i> AS ODN Anti- <i>ErbB-2</i> single-chain antibody	Prostate cancer (phase I) ^a 17% Complete responses and 33% partial responses in patients with malignant glioma (72) Malignant glioma, non-small-cell lung cancer ^a Metastatic breast cancer ^a Head and neck squamous cell carcinoma ^a 38% Stable disease and 61% progressive disease in ovarian carcinoma trial (638)
Suicide gene therapy	<i>HSV-TK</i> <i>CYP2B1</i> <i>CD</i> <i>Nitroreductase</i>	Occasional partial responses in phase I and II trials (36, 38, 81–85, 94, 294, 410, 639, 640), but no significant therapeutic benefit over radiotherapy in a phase III study in newly diagnosed patients with glioblastoma multiforme (86) No response in pancreatic carcinoma (641) Breast cancer (phase I) ^a Head and neck, liver, ovarian cancer (phase I) ^a
Combined approaches	IL-2 + GM-CSF; IL-7 + IL-2; IL-12 + B7-1; IL-7 + IL-12 + GM-CSF; IL-6 + sIL-6R; Muc-1 + IL-2; IL-2 + IFN- γ ; k-Ras AS ODN + p53; B7-1 + CEA; IL-2 + Lymphotactin; Staphylococcus Enterotoxin B + IL-2; CD + HSV-TK; IL-7 + HSV-TK; IL-2 + HSV-TK	Melanoma, lymphoma, renal cancer, colon cancer, myeloma, ovarian cancer, breast cancer, prostate cancer, non-small-cell lung cancer, neuroblastoma. ^a Partial responses in glioblastoma multiforme (88) and anaplastic thyroid carcinoma (642) with HSV-TK + IL-2 gene transfer
Chemoprotection	<i>MDR-1</i>	Metastatic cancer, lymphoma, germ cell cancer (643–645)
Oncolysis	<i>dl1520</i> adenovirus CN706 adenovirus Ad5-CD/TK rep adenovirus G207 herpesvirus 1716 herpesvirus Vaccinia GM-CSF Newcastle disease virus H-1 autonomous parvovirus Reolysin reovirus	Head and neck, liver, ovarian, pancreatic colorectal cancer (phase I–III). Occasional objective tumor responses in up to 25% of cases in phase I–II trials (30, 49–52, 54, 79, 96–99, 646, 647). Prostate carcinoma (phase I). Response in 65% (57) Prostate carcinoma (phase I). Response in 62.5% (648) Glioblastoma multiforme (phase I). Partial response in 40% (60) Glioblastoma multiforme, melanoma (phase I). Tumor response and long-term survival in 20–30% (61, 649, 650) Melanoma (phase I). Response in 70% (39) Advanced solid cancer (phase I). Response in 14% (68) Phase I clinical trials ^a Phase I clinical trials ^a

AS ODN, Antisense oligodeoxynucleotide; TK, thymidine kinase; CD, cytosine deaminase.

^a No available results from clinical trials.

the decision to put a clinical hold on similar trials in the same disease in the United States (15). Two of 11 boys treated so far in this clinical trial developed T cell leukemia-like illness about 3 yr after the gene therapy procedure, one in Septem-

ber 2002 and the other in December 2002. The first case involved the replication of a single clone of $\gamma\delta$ T cells, and the second involved an excess of three clones of $\alpha\beta$ T cells. In both cases, leukemia was probably a consequence of inser-

tional oncogenesis, because the vector inserted itself within or near the same gene, *LMO-2*, which has been linked to leukemia T cells (16–20). However, because the complication of leukemia has not occurred in any other clinical trial (20), multiple factors could have contributed to the development of leukemia in the patients involved in this trial. These include the high level of engraftment and expansion of the genetically modified cells, unique properties of the hematopoietic stem and progenitor cells in bone marrow of X-SCID patients, the immune deficiency of the X-SCID patients, and/or the transferred gene itself (19).

2. Safety in cancer gene therapy clinical trials. Although significant therapeutic benefit has not yet been demonstrated from cancer gene therapy clinical trials, a positive remark for gene therapy derives from the substantial safety of this approach in cancer patients treated so far (Table 2).

Most of the clinical trials of cancer have been based on the direct intratumor administration of recombinant vectors for therapeutic gene transfer, whereas systemic delivery of vectors has been more recently attempted. With regard to intratumor injection of vector particles or viral vector-producing cells, most studies demonstrate the safety of this approach, with only minor morbidity, generally related to the injection procedure. Mild pain and bleeding at the injection site have been reported in up to 50% of patients receiving intratumor inoculation of vectors (21–26). Pneumothorax has occurred in patients treated with pulmonary injections of vectors for advanced lung cancer or tumors metastatic to the lung (23, 27). Sterile peritonitis and pleuritis have been occasionally reported in the case of ip or intrapleural administration of viral vectors (28–30).

Different side effects are observed by using different types of vector, and toxicity is related to vector dose and site of administration. Fever and chills are common side effects observed after intratumoral administration of high doses of adenoviral vectors (31–35); subarachnoid hemorrhage and aseptic vasculitis may occur after intracerebral inoculation of retroviral vector-producing packaging cells for recurrent glioblastoma multiforme (36); confusion, hyponatremia, seizure, and signs of central nervous system toxicity occurred in patients with end-stage malignant brain tumor injected with adenoviral vectors (37, 38); and mild transient flu-like symptoms and local inflammation with pustule formation occurred after intratumoral injection of high doses of recombinant vaccinia virus vectors (39, 40). Administration of large doses of plasmid DNA appeared to be well tolerated without evidence of the development of anti-DNA antibodies (41). Mild to moderate toxicity was reported after direct intraleisional delivery of Allovectin-7, an HLA-B7/ β 2-microglobulin DNA-liposome complex, in patients with metastatic melanoma (23). Symptoms included pruritus and erythema at the injection site and general aches and pains (23).

With regard to adenoviral vectors, a revision of safety parameters and long-term follow-up in 102 subjects receiving local administration (*i.e.*, to the nasal and bronchial epithelium, metastatic tumors, skin, myocardium, and skeletal muscle) of low ($<10^9$ particle units) and intermediate (10^9 to 10^{11} particle units) doses of replication-deficient adenoviral vectors demonstrated an incidence of 0.7% major adverse

events, but no deaths related to an adenoviral vector (35). The incidence of malignancy was within that expected for the population. Most adverse events associated with administration of the vector to the respiratory epithelium were fever and/or leukocytosis associated with the bronchoscopy procedure, and, occasionally, increase in fibrinogen and liver function tests. Vector administration to colon cancer metastatic to the liver was sometimes accompanied by mild transient increase of transaminases, mild fever, leukopenia, and thrombocytopenia (35, 42). Mild transient increase in transaminases and transient hypotension were reported also after intratumor adenoviral vector administration in patients with mesothelioma (43, 44) or head and neck cancer (32). Analysis of risk factors for adverse events in patients treated with local delivery of adenoviral vectors demonstrated that vector-related parameters, including dose, route, transgene, or number of vector administrations, did not predict the occurrence of major adverse events (34).

Replication-competent viral vectors may show side effects related to local viral replication. Most clinical experience with replicating viruses has been achieved with the E1B-deleted adenovirus *dl1520*. Lacking E1B activity, this vector should selectively replicate, and thereby kill, in cells with unpaired p53 function (45), although replication in cells with wild-type p53 has been demonstrated (46–48). Several clinical protocols with this agent have been completed so far, including trials in patients with head and neck cancer (49), pancreatic adenocarcinoma (50), hepatocellular carcinoma (51, 52), ovarian cancer (30), and gastrointestinal malignancies metastatic to the liver (51, 53–55). Phase I studies were based on intratumoral injection of a single dose of *dl1520* ranging from 10^7 to 10^{11} plaque-forming units (pfu). All of these showed that escalation of virus dose was possible without dose-limiting toxicity. Most patients reported flu-like symptoms after *dl1520* administration. Symptoms generally started after the first virus dose and consisted of generalized malaise, headaches, nausea, myalgias, pyrexias, and rhinorrhea. Pain at injection site was reported in patients with head and neck cancer receiving intratumoral application of up to 10^{11} viral particles (49). Doses of *dl1520* ranging from 2×10^8 to 2×10^{12} pfu administered through the hepatic artery were well tolerated, with only transient fever and elevation of liver enzymes (51, 55). Dose-escalation was possible, and the maximum dose, which was based on manufacturing capabilities, was shown to be well tolerated in treated patients (55). Intravenous administration of up to 2×10^{12} *dl1520* viral particles was also well tolerated, except for mild to moderate constitutional symptoms and transient dose-dependent increase of serum aminotransferase (51, 54). Intraperitoneal injection of 10^9 to 10^{11} pfu for ovarian cancer was associated with abdominal pain, consistent with peritonism, diarrhea, heartburn, and vomiting (30). Interestingly, no cytopathic effects suggesting viral replication have been observed in normal tissues surrounding injected tumor tissues (55).

Other replicating viruses recently introduced to the clinic include the conditionally replicating adenovirus CV706, in which the adenoviral *E1A* gene is driven by prostate-specific antigen (PSA) promoter/enhancer elements and, therefore, it can selectively replicate in prostate tissue (56). A recently completed phase I/II trial in patients with locally recurrent

TABLE 2. Side effects of gene therapy in clinical trials for cancer

Protocol	Gene/vector/route	Phase	No. of patients	Tumor type	Side effects	Outcome	Ref.
Histocompatibility antigens	<i>HLA-B7</i> /liposome/intratumor	I	5	Stage IV melanoma	No	1 CR	651
	<i>HLA-B7</i> /β2-microglobulin/liposome/intratumor	I	9	Squamous cell carcinoma of the head and neck	No	4 PR	652
	<i>HLA-B7</i> /liposome/intratumor	II	52	Stage IV melanoma	Pain, hemorrhage, pneumothorax, hypotension	1 CR, 3 PR, 5 MR	23
	Human and murine MHC/liposome/intratumor	I/II	19	Cutaneous metastases	No	2 local CR, 4 local PR	653
	<i>IL-2</i> /adenovirus/ <i>ex vivo</i> /sc injection	I	15	Stage IV melanoma	Erythema, induration, and pruritus at injection site in all patients; flu-like symptoms in some patients	3 PR	654
Immunotherapy and vaccination	<i>IL-2</i> /adenovirus/intratumor	I	23	Stage IV melanoma	Local inflammation in 15, fever in 5	7 local responses	655
	<i>IL-2</i> /adenovirus/intratumor followed by prostatectomy	I	12	Localized prostate cancer	Lymphopenia in 1 patient, perineal discomfort in most patients, hematuria and flu-like symptoms in 2 patients	Significant decrease of PSA levels in most patients	656
	<i>IL-2</i> /adenovirus/intratumor	I	21	NSCLC	NR	NR	657
	<i>IL-2</i> autologous tumor cells/ <i>ex vivo</i> /sc injection	I	12	Stage IV melanoma	Mild fever and headache	3 SD	658
	<i>IL-2</i> in allogenic tumor cell line/ <i>ex vivo</i> /sc injection	I/II	33	Stage IV melanoma	Swelling of locoregional lymph nodes, induration at injection site	3 Regression of distant metastases; 2 CR; 7 SD	659
	<i>IL-2</i> autologous cytokine-induced cells/ <i>ex vivo</i> /fv	I	10	Metastatic renal cell carcinoma, colorectal carcinoma, lymphoma	Fever in 3	1 CR (lymphoma), 3 SD, 6 PD	660
	<i>IL-2</i> in autologous fibroblasts + autologous tumor cells/ <i>ex vivo</i> /sc injection	I	10	Colorectal carcinoma	Fatigue and flu-like symptoms in 7, delay-type hypersensitivity skin reactions in 5	1 SD, 9 PD	661
	<i>IL-2</i> /liposome/intratumor	I	24	Melanoma (7), sarcoma (4), colorectal cancer (4), renal cell carcinoma (3), and others	Mild constitutional symptoms in 10, pain at injection site in 16, hemorrhage at injection site in 5, pneumothorax in 3	5 Minor responses, 4 SD	21
	<i>IL-2</i> in allogenic tumor cell line/ <i>ex vivo</i> /sc injection	I/II	52	Melanoma (18), renal cell carcinoma (17), sarcoma (17)	Mild constitutional symptoms in 27, grade 3 rigor in 1, pain at injection site in 23	3 PR (2 renal cell carcinoma, 1 melanoma), SD (2 renal cell carcinoma, 3 melanoma, 6 sarcoma)	21
	<i>IL-2</i> in xenogeneic fibroblasts (Vero- <i>IL-2</i> cells)/ <i>ex vivo</i> /sc injection	I	9	Metastatic solid tumors	Fever in 1, mild itching and erythema in 2	1 PR, 4 SD	26
Tumor suppressor gene therapy	<i>IL-7</i> /gene gun/ <i>ex vivo</i> /sc injection	I	10	Stage IV melanoma	Mild fever	Minor response in 2	74
	<i>IFN-γ</i> /retrovirus/ <i>ex vivo</i> /sc injection	I	5	Stage IV melanoma	No	1 Long-term disease-free	75
	<i>p53</i> /adenoviral/intratumor	I	21	Advanced NSCLC	Mild fever	Amelioration of time to disease progression	634
	<i>P53</i> /adenoviral/intratumor + chemotherapy	I	24	Advanced NSCLC	Transient fever in 8, injection site pain in 1	2 PR, 17 SD, 4 PD, 1 NE	90
	<i>P53</i> /adenoviral/intratumor	I	15	Advanced NSCLC	No	4 SD	635
	<i>P53</i> /adenoviral/intratumor + chemotherapy	II	25	Advanced NSCLC	Mild fever, flu-like symptoms, nausea or anorexia, fatigue; occasionally, injection-site complications	No significant benefit over chemotherapy alone	31
	<i>p53</i> /adenoviral/intratumor or intravesical instillation + cystectomy	I	12	Bladder cancer	Urethral or vesicle burning	Not evaluated	25
	<i>p53</i> /adenoviral/intravesical instillation	I	13	Bladder cancer	Bladder spasm in 3, urothelial ulceration in 1	2 SD, 11 PD	662

Protocol	Gene/vector/route	Phase	No. of patients	Tumor type	Side effects	Outcome	Ref.
	<i>P53</i> /adenoviral/iv + chemotherapy	I/II	36	Recurrent ovarian cancer	Fever, hypotension, abdominal pain, nausea, vomiting	>50% reduction of CA125 levels in 8; 10 long-term survivors after treatment with multiple doses	636, 637
	<i>P53</i> /adenoviral/intratumor	I	27	Advanced NSCLC	Minimal toxicity	2 PR, SD in 16, 7 PD	27
	<i>P53</i> /adenoviral/intratumor + radiotherapy	II	19	Advanced NSCLC	Grade 1-2 fever (79%) and chills (53%)	1 CR, 11 PR, 3 SD, 2 PD, 2 NE	95
	<i>P53</i> /adenoviral/intratumor + surgery	I	15	Recurrent glioma	Grade 3 hemiparesis in 1 patient, aphasia in 2; headache in 53% of patients, fatigue in 40%, fever in 27%	Mean progression-free survival 13 wk, median overall survival 43 wk	663
	<i>P53</i> antisense oligonucleotide/iv	I	16	Hematologic malignancies	No	No response	664
	<i>BRCA1</i> /retrovirus/iv	I	12	Ovarian cancer	3 Acute sterile peritonitis	3 PR, 8 SD	28
	<i>BRCA1</i> retrovirus/iv	II	6	Ovarian cancer	3 Acute sterile peritonitis	6 PD	80
	<i>IGF-IR</i> antisense oligonucleotide treatment of tumor cells/ <i>ex vivo</i> /sc implantation	I	12	Anaplastic astrocytoma	4 Deep venous thrombosis	2 CR, 4 PR	72
Oncogene suppression	Anti-erbB2 single chain antibody/adenovirus/iv	I	15	Ovarian cancer	9 Transient fever	5 SD, 8 PD	638
	<i>HSV-TK</i> /retrovirus/intratumor	I	5	Recurrent glioblastoma	No	1 PR	82
Suicide gene therapy	<i>HSV-TK</i> /retrovirus/intratumor	I	15	Recurrent glioblastoma	No	3 PR, 1 CR	81
	<i>HSV-TK</i> /retrovirus/intratumor	I	12	Recurrent glioblastoma	3 Serious infectious events and 1 subarachnoid hemorrhage related to the surgical procedure	4 SD	36
	<i>HSV-TK</i> + <i>IL-2</i> /retrovirus/intratumor	I/II	14	Recurrent glioblastoma	No	1 CR, 5 PR, 4 SD, 4 PD	Palù <i>et al.</i> (unpublished data) and Ref. 84
	<i>HSV-TK</i> /retrovirus/intratumor + surgery	I/II	48	Recurrent glioblastoma	Seizures in 1, hydrocephalus in 1	4 PR	38
	<i>HSV-TK</i> /adenovirus/intratumor	I	13	Recurrent glioblastoma	Transient central nervous system adverse effects in 2	3 SD	85
	<i>HSV-TK</i> /retrovirus/intratumor after surgery	I	12	Brain tumors	Transient central nervous system adverse effects in 4	Not reported	83
	<i>HSV-TK</i> /retrovirus or adenovirus/intratumor	I/II	14	Recurrent glioblastoma	Fever in 2, epileptic seizures in 2	7 PD with retroviral vector, 3 SD and 4 PD with adenoviral vector	86
	<i>HSV-TK</i> /retrovirus/intratumor	III	124	Glioblastoma	Cerebral hematomas, thromboembolism, complications associated with the presence of central venous catheter for GCV infusion (pneumothorax, thrombosis, infection)	Nonsignificant differences with control group	665
	<i>HSV-TK</i> /adenovirus/intratumor after surgery	I	14	Recurrent glioblastoma	Postoperative aphasia and emiparesis, cerebrospinal fluid leak, thrombocytopenia, sepsis	No clinical response	294
	<i>HSV-TK</i> -[¹²⁴ I]-FIAU/hiposome/intratumor	I/II	5	Recurrent glioblastoma	No	1 PR	639
	<i>HSV-TK</i> /adenovirus/intratumor	I	18	Prostate carcinoma	Minimal toxicity in 4, severe thrombocytopenia and hepatotoxicity in 1	PSA response in 1	94
	<i>HSV-TK</i> /adenovirus/intratumor + radiotherapy	I/II	30	Prostate carcinoma	Flu-like symptoms in 11, fever in 6, mild hepatotoxicity in 14	Not reported	640
	<i>HSV-TK</i> /adenovirus/intratumor	I	11	Prostate carcinoma	No	1 SD	410
	<i>HSV-TK</i> /retrovirus/intratumor	I/II	8	Stage IV melanoma	Inflammation at injection site	8 PD	G. Palù <i>et al.</i> (unpublished data)
	<i>HSV-TK</i> + <i>IL-2</i> /retrovirus/intratumor	I	2	Anaplastic thyroid carcinoma	No	2 SD	641
	<i>CYP2B1</i> in HEK293 cells/ <i>ex vivo</i> /iv encapsulated cells	I/II	14	Pancreatic carcinoma	No	2 PR, 2 MR, 10 SD	

Protocol	Gene/vector/route	Phase	No. of patients	Tumor type	Side effects	Outcome	Ref.
Oncolysis	d/1520adenovirus/intratumor	I	22	Recurrent head and neck cancer	Fever, nausea, chills	3 PR	97
	d/1520 adenovirus/intratumor	I	3	Hepatocellular carcinoma	No	Not reported	51
	d/1520 adenovirus/intrahepatic artery or iv	I	6	Colon cancer liver metastases	1 mild shivers, 1 fever	Not reported	51
	d/1520 adenovirus/intrahepatic artery + chemotherapy	II	7	Liver metastases from colon cancer (6) or unknown primary (1)	2 mild shivers, 1 fever	6 SD, 1 PD	51
	d/1520 adenovirus/iv on d 1 and intratumor on subsequent days	II	5	Hepatocellular carcinoma	3 transient fever, 2 hypotension	1 PR, 4 PD (2 SD, 3 PD in control group treated with percutaneous ethanol injection)	52
	d/1520 adenovirus/intratumor	I	23	Pancreatic carcinoma	Flu-like symptoms, fever	6 MR, 10 SD	50
	d/1520 adenovirus/intratumor + chemotherapy	I	10	Metastatic solid tumors	Fever, rigor	1 MR, 8 SD	54
	d/1520 adenovirus/iv	I	16	Advanced ovarian cancer	Flu-like symptoms, fever, emesis, abdominal pain, diarrhea	4 SD	30
	d/1520 adenovirus/intratumor + chemotherapy	I/II	21	Advanced pancreatic carcinoma	Mild flu-like symptoms, transient lipase elevation, 2 sepsis, 2 duodenal perforations from endoscopic tip	2 PR, 2 minor response, 6 SD, 11 PD	646
	d/1520 adenovirus/intratumor	II	37	Recurrent head and neck cancer	Injection site pain, flu-like symptoms	8 CR, 1 PR	49
	d/1520 adenovirus/intratumor + chemotherapy	II	40	Head and neck cancer	Mild to moderate fever (67%), injection site pain (47%)	3 CR, 2 PR, 3 minor response, 13 SD, 15 PD	647
	d/1520 adenovirus/intrahepatic artery + chemotherapy	II	27	Gastrointestinal carcinoma metastatic to the liver	Mild to moderate flu-like symptoms in 25, systemic inflammatory reaction and transient severe hepatotoxicity in 1	3 PR, 4 moderate response (including 3 chemotherapy-resistant tumors), 9 SD, 11 PD	99
	CN706 adenovirus/intratumor	I	20	Recurrent prostate cancer	Mild to moderate fever in 15, pain and inflammation at injection site in 14, microscopic hematuria in 20	Reduction in serum PSA > 30 in 13 (including 4 PR)	57
	Ad5-CD/TK rep adenovirus	I	16	Recurrent prostate cancer	Injection site pain and inflammation (80%), flu-like symptoms and fatigue (40%)	7 Minimal responses, 3 PR	648
	γ34.5 null/G207 herpesvirus/intratumor	I	21	Recurrent glioblastoma (16) and anaplastic astrocytoma (5)	No	8 PR	60
	γ34.5, UL39 null/1716 herpesvirus/intratumor	I	9	Recurrent glioblastoma (8) and anaplastic astrocytoma (1)	No	5 SD, 4 PD	61
	γ34.5, UL39 null/1716 herpesvirus/intratumor	I	12	Recurrent glioblastoma (11) and anaplastic astrocytoma (1)	Cerebral hemorrhage in 1	1 CR, 3 PR	649
	γ34.5, UL39 null/1716 herpesvirus/intratumor	I	5	Metastatic melanoma	No	1 PR	650
	Vaccinia-GM-CSF/intratumor	I	7	Metastatic melanoma	Flu-like symptoms, local inflammation	1 CR, 1 PR, 3 MR, 2 PD	39
	PV701 Newcastle disease virus/iv	I	79	Advanced solid cancer (23 colorectal, 9 pancreatic, 9 renal, 8 breast, 8 NSCLC, 4 sarcoma, 4 head and neck, 3 melanoma, etc.)	Fever, flu-like symptoms, hypotension, vomiting and diarrhea, acute dose reactions (back pain, chest tightness, chest pain, hypotension), inflammation and adverse events at tumor site	62 evaluable patients: 1 CR, 1 PR, 7 minor response, 14 SD	68

CR, Complete response; PR, partial response; MR, mixed response; SD, stable disease; PD, progressive disease; NSCLC, non-small-cell lung cancer.

prostate cancer showed that doses of up to 10^{13} particles of the CV706 vector, administered using brachytherapy techniques, appear to be safe, although biochemical (PSA) responses were observed in a minority of patients (57). A more potent oncolytic adenovirus, CV787, which contains the prostate-specific rat probasin promoter driving *E1A* expression and the human prostate-specific enhancer/promoter driving the *E1B* gene (58), is currently being studied in phase I and II clinical trials. This virus, unlike CV706, maintains a wild-type E3 region, which encodes proteins that play a role in cell lysis and evasion of host immune response (59). Other oncolytic agents currently under evaluation in phase I and II clinical trials include replication competent G207, HSV1716, and NV1020 herpes simplex viruses (HSV). The double mutant G207 HSV harbors deletions of both copies of the $\gamma 34.5$ gene and contains an insertional inactivation of the ICP6 gene, which encodes a subunit of viral ribonucleotide reductase. The virus has been administered stereotactically in patients with recurrent gliomas at doses up to 3×10^9 pfu without any significant toxicity being encountered (60). In particular, no patient has developed HSV encephalitis (60). The HSV1716 virus, which lacks both copies of $\gamma 34.5$ has been safely administered intratumorally up to doses of 10^5 pfu in patients with recurrent high-grade glioma (61, 62) or metastatic melanoma (63). NV1020 is currently being evaluated in a phase I trial as a vaccine in the treatment of patients with colorectal carcinoma liver metastases. This virus has only one copy of $\gamma 34.5$ deleted and maintains sensitivity to acyclovir and ganciclovir (GCV). In addition, it contains an exogenous copy of the thymidine kinase gene under control of the powerful HSV-1 $\alpha 4$ promoter. Genetic stability and safety have been demonstrated in extensive rodent and primate studies as well as in limited human vaccine trials (64, 65). Other replicating viruses currently under evaluation in phase I/II clinical trials include reovirus, a virus that replicates in malignant cells with activation of the Ras signaling pathway (66), the animal pathogen Newcastle disease virus (63, 67), vaccinia virus (39, 69), and autonomous parvoviruses (70, 71). Preliminary results from clinical trials are encouraging, and no serious adverse events have been demonstrated so far. The most common adverse events were flu-like symptoms occurring principally after administration of the first dose of the Newcastle disease virus PV701 (68) or after intratumoral injection of vaccinia/granulocyte macrophage-colony stimulating factor (GM-CSF) recombinant virus (39).

Side effects directly related to therapeutic transgenes are less frequent, such as in the case of mild flu-like symptoms observed after administration of *IL-2* cDNA in liposome complex (21), or in the case of fever, fatigue, or change in mental status in patients receiving intratumor injection of the *E1A* adenovirus gene as a lipid complex (24). Deep vein thrombosis was reported in patients with malignant astrocytoma, who underwent implantation of autologous glioma cells, treated *ex vivo* with an antisense oligodeoxynucleotide directed against the IGF type I receptor (IGF-IR) (72). Side effects due to the prodrug used in suicide gene therapy also have been reported, *i.e.*, rise of liver enzymes after GCV administration (43).

3. *Efficacy in cancer gene therapy clinical trials.* Despite anecdotal reports of therapeutic responses in several patients, unequivocal proof of the clinical efficacy of cancer gene therapy is still lacking (Tables 1 and 2).

Of the different approaches to cancer gene therapy, including immunotherapy, tumor suppressor gene replacement, and suicide gene/prodrug activation therapy, immunotherapy showed better clinical results, being less affected by the limitations related to vector titer and transduction efficiency. Partial responses were observed after *IL-2* (21, 26), *HLA-B7* (23, 73), *IL-7* (74), or *GM-CSF* (39) gene transfer in patients with advanced solid tumors, including renal cell carcinoma, melanoma, and soft-tissue sarcomas. At variance, no evidence of tumor response was seen at sites distal from the injected tumor in a phase I trial of interferon- γ (IFN- γ) retroviral vector administered intratumorally to patients with metastatic melanoma (75).

Clinical and radiological improvements were observed in patients with malignant astrocytoma, after *ex vivo* treatment of autologous tumor cells with an antisense oligodeoxynucleotide directed against the IGF-IR (72). Minor tumor responses also were demonstrated in two of 16 evaluable patients with recurrent breast and head and neck cancer, receiving intratumoral liposome *E1A* gene therapy (24). In this strategy, the *E1A* adenovirus gene functions as a tumor suppressor gene by inhibiting expression of *HER-2/neu* and other oncogenes, inducing apoptosis in cancer cells and sensitizing cancer cells to chemotherapeutic drugs (76, 77).

Using tumor suppressor gene-replacement approaches, transient local disease control and partial tumor responses were observed after viral vector-mediated delivery of wild-type *TP53* in phase I and pilot studies in patients with lung cancer (27, 78, 79), head and neck cancer (32), bladder cancer (25), or metastatic malignant liver tumor (51). However, a controlled phase II study in patients with newly diagnosed advanced non-small-cell lung cancer failed to demonstrate a significant clinical benefit from local *TP53* gene transfer by intratumoral vector injection in combination with effective first-line chemotherapy (31). A phase I clinical trial in end-stage ovarian cancer patients treated with ip administration of retroviral vectors expressing the *BRCA1* tumor suppressor gene reported tumor reduction in three of 12 treated patients, vector stability, and minimal antibody response (29). At variance, a subsequent phase II protocol on six patients with less extensive disease showed no response, no disease stabilization, and rapid clearance of the vector due to antibody development (80). Conceivably, patients' immune system status played a major role in conditioning gene therapy effectiveness.

Regarding prodrug activation therapy, phase I/II studies in patients with recurrent brain tumors receiving intratumor stereotactic administration of packaging cells producing a retroviral vector encoding for the thymidine kinase gene of HSV type 1 (HSV-TK), followed by treatment with GCV reported up to 30% objective responses (36, 38, 81–85). However, no significant therapeutic benefit over radiotherapy was obtained in a phase III study in newly diagnosed patients with glioblastoma multiforme (86). A combined approach, based on stereotactic intratumor injection of packaging cells producing a retroviral vector carrying the human

IL-2 and the *HSV-TK* genes (87), followed by GCV administration, led to tumor regression in four treated patients, with partial response in one case (88).

An improvement of gene therapy efficacy has been observed in association with conventional radiotherapy and chemotherapy. Chemosensitization of a variety of cancers after wild-type *TP53* delivery has been demonstrated in *in vitro* and *in vivo* preclinical studies (89) and confirmed in a clinical trial in patients with non-small-cell lung cancer treated with a nonreplicating p53 adenoviral vector (90). Radiosensitization has also been demonstrated after *TP53* or *HSV-TK* gene transfer (91–94). A phase I/II trial of radiation therapy in combination with three biweekly intratumor injections of p53 adenoviral vector in patients with advanced non-small-cell lung cancer documented a 1-yr progression-free survival of 45.5%, superior to historic controls (27). These results have been recently confirmed in a phase II protocol, reporting a response rate of 12 of 19 treated patients (95).

First results from clinical trials with conditionally replicating oncolytic viruses are available. In phase I/II dose-escalation protocols of intratumoral injection of *dl1520*, objective tumor responses, generally minor, were reported in 10–25% of cases, even at high virus doses (96). In particular, tumor necrosis at the site of single *dl1520* injection was demonstrated in three of 22 patients with head and neck cancer enrolled in a phase I trial (97), whereas in a subsequent phase II study of repeated virus injection, three complete responses and two partial responses were observed out of 40 treated patients (79). Mild tumor responses also were reported in six of 23 patients with pancreatic adenocarcinoma enrolled in a phase I study of intratumor administration of *dl1520* (98).

Patients who received the highest viral doses (10^{12} pfu) experienced better survival than patients treated with the lower doses in a phase I study of intraarterial *dl1520* administration in patients with colorectal carcinoma liver metastases (99). On the other hand, no significant tumor response was achieved with *dl1520* as a single therapeutic agent in patients with hepatocellular carcinoma (52), recurrent ovarian cancer (30), or advanced solid cancers metastatic to the lung (54).

Treatment with the conditionally replicating HSV mutant G207 at doses of 10^6 to 3×10^9 pfu led to a decrease in tumor volume in eight of 20 patients with recurrent malignant gliomas enrolled in a phase I trial, including two long-term survivors (60, 100). In a phase I study of replication competent HSV1716 at doses of 10^3 to 10^5 pfu in nine patients with recurrent malignant gliomas, four cases of long-term survival were documented, although no tumor responses were detected (61). Two complete tumor responses and three partial responses were observed, with evidence of viral replication and immune infiltration in injected lesions in a phase I clinical protocol of intralesional administration of replication competent vaccinia virus carrying the *GM-CSF* gene in patients with refractory, recurrent melanoma (39).

Conventional chemotherapy and radiotherapy also have been associated with delivery of replication-competent viruses, resulting in improved clinical response. In a clinical trial in head and neck cancer patients treated with *dl1520* as a single therapeutic agent, objective responses were observed in 15% of cases compared with 60% of patients when the

treatment was combined with 5-fluorouracil/cisplatin chemotherapy (49). Stabilization of disease and two cases of objective response were achieved in patients with multiple colorectal liver metastases undergoing intraarterial *dl1520* and 5-fluorouracil infusion (51) or intrahepatic artery *dl1520* infusion plus iv 5-fluorouracil and leucovorin (55).

4. *Comment.* A decade of clinical trials for cancer has demonstrated disappointing results, with minimal antitumor efficacy of currently available gene therapy tools. On the other hand, treatment modalities have been demonstrated to be safe, with only minor gene therapy-related toxicities. On a cost-benefit analysis, even anecdotal reports of cases of response to gene therapy in patients with tumors refractory to conventional treatment still favor gene therapy intervention, considering the substantial safety of the procedure.

Assessment of transgene expression in target cells has demonstrated poor transduction efficiency of gene transfer vectors, which conceivably accounts for most therapeutic failures. Thus, key issues to be considered are the improvement of vectors to achieve high levels of therapeutic gene expression and transduction of a sufficient number of target cells to result in clinical benefits. To overcome the need to infect all tumor cells to achieve complete response, suicide or cytokine genes should be inserted into oncolytic vectors to increase tumor cell killing and antitumor immunity.

B. Gene therapy strategies for targeting endocrine and endocrine-related tumors

1. *Overview.* 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 minimize 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* to achieve therapeutic levels of transduction.

Targeting of vectors can be obtained in many ways (Table 3). The easiest one is to administer the vector directly at the target site. For systemic administration, molecular engineering is required to target either gene expression (transcriptional targeting) or gene delivery (transductional targeting). Transcriptional targeting can be attempted by the introduction of tissue-specific or tumor-specific enhancers/promoters that control the expression of therapeutic genes (101, 102). Transductional targeting is based on enhanced interaction between the vector and target cell surface. It may exploit the natural tropism shown by some viruses for specific tissues or be achieved by modification of viral envelope protein sequences, by insertion of ligand molecules, by viral envelope pseudotyping, or by expression of antibodies on the viral particle surface to confer new binding specificity toward target cell receptors (103).

Endocrine glands appear to favor gene therapy targeting at different levels: 1) the easily accessible anatomical site of some endocrine glands (thyroid, pituitary) allows the direct inoculation of the vector and the evaluation of cell transduction; 2) the transcriptional control elements (enhancer/promoter) responsible for expression of tissue-specific genes

TABLE 3. Targeting strategies for cancer gene therapy

Targeting strategies	Example (Refs.)
Transductional targeting	
Pseudotyping	Pseudotyping of murine leukemia virus-based retroviral vectors with VSV envelope glycoprotein G (666)
Engineering of viral surface proteins	Engineering envelope glycoproteins of retroviral vectors (103, 667, 668) Modification of the fiber protein of adenoviral vectors (669) Modification of the penton base of adenoviral vectors (670) Engineering of HSV vector envelope proteins (671)
Retargeting by use of bifunctional crosslinkers	Bifunctional antibodies (103) Soluble receptors and adapters (377)
Transcriptional targeting	
Tissue-specific promoters	PSA (672) Tyrosinase (673)
Tumor-specific promoters	Telomerase (674) c-erbB2 (675) c-Myc (676)
Inducible promoters	Early growth response gene (EGR-1) promoter (677) Hsp70 (217) MDR-1 (678)
Transcriptional regulation of viral replication	E1A controlled by DF3/MUC1 promoter (174) ICP4 controlled by albumin enhancer/promoter (679)

(hormones, hormone receptors) may be used to selectively direct transgene expression; 3) tissue-specific surface proteins (such as hormone receptors) may be used as a target for vectors with modified tropism (104).

2. *Transductional targeting approaches.* Attempts to improve efficacy and selectivity of vector targeting to endocrine and endocrine-related tumor cells have been reported only for breast, ovary, and prostate cancer. An example of vector retargeting was the engineering of retroviral vector envelope glycoproteins were to display single chain antibodies recognizing Her2/*neu*, which is a member of the epidermal growth factor (EGF) receptor (EGFR) family of receptors, overexpressed in 20–30% of breast and ovarian cancers (105). Targeting of the EGFR in tumor cells was also performed with adenoviral vectors, by using a bifunctional crosslinker, *i.e.*, the Fab fragment of an antiknob monoclonal antibody conjugated with an anti-EGFR monoclonal antibody (106), or by using a single chain Fv antibody fragment specific to the fiber, linked to EGF as a fusion protein (107). Other efforts of adenoviral retargeting by using bifunctional crosslinkers include conjugates between antiviral knob monoclonal Fab fragment and fibroblast growth factor (108) or folate (109) to target fibroblast growth factor receptor and folate receptor, respectively, both overexpressed on the surface of a variety of tumor cells, including ovarian and breast carcinoma cells.

An alternative transductional targeting approach is based on engineering of surface viral molecules to modify viral tropism. An example of this approach is the generation of a modified AAV vector displaying a 15-amino acid peptide, which binds to the human LH receptor (LH-R), to selectively transduce LH-R-bearing cells, such as ovarian cancer cells (110). Transduction was shown to be LH-R-mediated and to be increased by progesterone treatment, via induction of LH-R expression (110). Similarly, the fiber of an oncolytic adenovirus was modified by incorporating an integrin binding motif to increase transduction of ovarian cancer cells, which generally do not express the coxsackie adenovirus receptor (111, 112). At variance with adenoviral vectors, engineering of envelope glycoproteins of ecotropic and am-

photropic retroviruses to redirect virus tropism may markedly impair transduction efficiency, such as is the case of modified retroviruses targeting EGFR, IGF receptor, and folate receptor (113–115). It is conceivable that retargeted retroviral particles bind to the target cells in an envelope-independent manner and that the modification of cellular factors incorporated into the lipid envelope plays a dominant role in promoting initial adsorption of viral particles to cells. The receptor binding domain of the envelope glycoprotein would then function in a secondary recognition step essential for intracellular translocation of the virus particle.

Hypothetically, transductional targeting strategies could be feasible in a variety of endocrine tumors, exploiting targeting moieties such as antibodies and hormones directed to receptor molecules selectively expressed on the surface of the target endocrine tumor cell. Moreover, innovative methodologies, involving screening of phage display libraries, are available to find specific ligands with a high degree of specificity for the target cancer cell, without requiring that the molecules against which they are targeted be identified (116, 117).

3. *Transcriptional targeting approaches.* Transcriptional targeting strategies have been used largely to selectively express cytokine or suicide genes in endocrine and endocrine-related tumor cells (Table 3). Targeting has been attempted by using enhancer/promoter sequences of genes that are selectively expressed in endocrine and endocrine-related tissues or tumors. Endocrine tumors favor this targeting approach because, typically, they express a variety of specific genes.

a. Thyroid cancer. Transcriptional targeting of differentiated thyroid carcinomas was achieved by using thyroglobulin (TG) promoter to control therapeutic gene expression in either retroviral or adenoviral vectors (118–120). Strategies to enhance promoter activity included the use of a synthetic TG enhancer/promoter sequence (121) or a tandemly repeated TG promoter in an adenoviral vector (122), the replacement of viral enhancer with TG enhancer in a retroviral vector (123), or the use of histone deacetylase inhibitors (121, 124).

Another strategy to improve TG promoter activity was the use of a Cre-loxP system, in which the Cre recombinase was controlled by TG promoter to switch on transgene expression in target thyroid carcinoma cells (125). With regard to undifferentiated and anaplastic thyroid carcinomas, which do not produce TG, targeting of transgene expression was obtained by coexpressing thyroid transcription factor-1, which activates the TG promoter, together with TG promoter-driven therapeutic genes (126).

A tissue-specific approach was attempted also for medullary thyroid carcinomas, exploiting the promoter sequence of the calcitonin gene/calcitonin gene-related peptide gene to drive selective expression of therapeutic genes in target tumor cells (127). As for the TG promoter, also in the case of transcriptional control elements of the calcitonin gene/calcitonin gene-related peptide gene, increased efficacy and specificity were achieved by engineering a chimeric sequence containing a tandemly repeated enhancer sequence and a minimal promoter (128, 129).

b. Pituitary adenomas. Targeting therapeutic genes to specific cell types is particularly relevant for gene therapy of pituitary adenomas to spare normal pituitary cells and neighboring tissues. Cell type-specific expression of the therapeutic gene was achieved using the promoters of the GH, glycoprotein hormone α -subunit, prolactin (PRL), and POMC genes (130–134). As for thyroid tumors, also in the case of pituitary adenomas, Cre-mediated activation of loxP-repressed transcriptionally targeted therapeutic genes was demonstrated to be an efficacious strategy for targeted suicide gene therapy. In an *in vitro* and *in vivo* model of GH-secreting adenomas, coinfection with adenoviruses carrying either Lox-P-repressed diphtheria toxin gene under GH promoter regulation or the Cre recombinase gene under the control of GH promoter caused a marked tumor regression (135). In addition to cell type-specific expression of the therapeutic gene, regulated transgene expression was pursued by using a pharmacologically regulated gene expression system, such as the tetracycline-inducible system. By driving the expression of the tetracycline transactivator through the PRL-specific promoter, expression of the inducible transgene was restricted to both lactotrophic tumor cell lines and PRL-positive cells in primary anterior pituitary cultures and within the pituitary gland *in vivo* (136).

c. Adrenocortical carcinoma. Transcriptionally targeted gene therapy for adrenocortical carcinoma was attempted by using a chimeric enhancer/promoter element, containing both the *CYP11B1* promoter and the *P450SCC* enhancer to drive transgene expression (137). In this tumor model, expression of HSV-TK in stably transfected cells was enhanced by treatment with factors acting through the cAMP pathway, such as ACTH (137).

d. Prostate carcinoma. Due to the presence of well-characterized prostate-specific markers, such as PSA and a variety of prostate-unique genes, prostate carcinoma has represented an ideal model for targeted gene therapy treatment (138). Several studies used PSA regulatory regions to drive expression of therapeutic genes (139–149). Tandem duplication of the PSA enhancer increases expression approxi-

mately 50-fold while retaining tissue-specific control (150). A higher efficiency was achieved by coupling the PSA promoter to a yeast promoter (142, 151) or by complex engineering of the enhancer sequence (152). A minimal composite PSA promoter/enhancer element was used to drive expression of adenoviral E1A in the attenuated replication-competent vector CN706 (56, 153). The PSA enhancer region used in this vector contained a functional androgen response element capable of up to 100-fold induction of transgene expression in PSA-expressing cells in the presence of testosterone or the steroid analog R1881 (154). At variance, a long PSA promoter allowed efficient transgene expression both in the presence and absence of androgens (143). Coexpression of a partial androgen receptor gene and PSA-driven therapeutic gene allowed activation of the PSA enhancer/promoter even in the absence of androgens (155). Other tissue-specific promoters used to target prostate cancer include the human kallikrein gene promoter, which is expressed predominantly in the prostate and transcriptionally up-regulated by androgens (156, 157); prostate-specific membrane antigen, which is highly expressed in metastatic or poorly differentiated prostate cancer and up-regulated by androgen deprivation (158–161); the probasin promoter, selectively expressed in prostate cells (162–165); or the osteocalcin promoter to target metastatic lesions to the bone (166, 167). Tissue-specific, inducible systems were developed by using a prostate-specific chimeric promoter, based on the probasin gene promoter and two copies of the androgen response region, which was induced by activation of caspase after administration of a chemical inducer of dimerization (168). The same chimeric promoter sequence was used in a tetracycline-regulated expression system (169).

e. Breast carcinoma. Transcriptional targeting of the mammary tissue has been pursued by using either the human α -lactalbumin or ovine β -lactalbumin promoter to drive therapeutic gene expression (170). Tumor targeting was attempted by using promoters of tumor-specific genes, such as the *DF3/MUC-1* gene, which encodes a high molecular weight mucin-like glycoprotein overexpressed in the majority of breast cancers (171–174), and the *HER-2/neu* oncogene (also named *c-erbB-2*), which is overexpressed in a variety of human cancers, including breast and ovarian carcinomas (173, 175–177). A clinical trial was conducted for patients with recurrent breast carcinoma expressing the *HER-2/neu* gene (178). These patients were treated by intratumor injection of a plasmid containing the cytosine deaminase gene driven by the tumor-specific *erbB-2* promoter. Efficiency of cancer cell killing was proportional to cellular *HER-2/neu* expression.

Estrogen-responsive elements, which allow modulation of transgene expression by estrogens and tamoxifen, have been used to develop conditionally replicating adenoviral vectors to target estrogen receptor (ER)-positive breast cancer (179), or, in combination with hypoxia-responsive elements, to develop a targeted and regulated adenoviral vector (180). Gene therapy for breast carcinoma may also be approached by tailoring a virus with affinity to this tissue, such as the mouse mammary tumor virus. The glucocorticoid-responsive long terminal repeats (LTRs) of this retrovirus have been used as

promoters for dexamethasone-inducible oncolytic cytokine expression (181).

f. Ovarian carcinoma. Several tumor-specific or tissue-specific promoters have been investigated to target gene delivery to ovarian cancer. These include the promoter/enhancer sequences of the secretory leukoproteinase inhibitor gene (182, 183), the human epithelium-specific *ets* transcription factor gene *hESE1* (184), the *L-plastin* gene (185), and the *MUC1/DF3* gene (186), which are highly expressed in several epithelial tumors, including ovarian cancers.

g. Neuroendocrine tumors. After positive results from clinical studies of immunoscintigraphy and radioimmunotherapy with targeted monoclonal antibodies, several tumor/tissue-specific transcriptional regulatory sequences or antigens are being exploited to target transgene expression or vector delivery to neuroendocrine tumors. Tissue/tumor-specific genes under investigation include chromogranin A and B (187–191), calcitonin (192), neuron-specific enolase (193), arginine vasopressin (194), somatostatin receptor (195), and the *RET* protooncogene (196).

4. Comment. A number of possibilities to target endocrine and endocrine-related tumors by means of either transductional or transcriptional targeting are available and make these tumors ideal candidates for gene therapy. However, because malignant transformation is generally accompanied by cell dedifferentiation and loss of tissue-specific features, targeting is often not feasible. It is thus conceivable that effective endocrine tumor targeting could be achieved only by using oncolytic viruses characterized by selective tropism for endocrine glands. Indeed, as discussed in the following sections, several viruses have been shown to preferentially infect and replicate in endocrine cells, such as reovirus in thyroid and pancreas, adenovirus in adrenal cortex, and HSV in adrenal cortex and adrenal medulla. These viruses could be engineered as oncolytic agents for endocrine tumors or, more generally, for gene therapy applications in the endocrine system.

II. How to Exploit the Endocrine System for Regulating Therapeutic Gene Expression

The first clinical applications of cancer gene therapy did not require fine regulation of transgene expression, either because of short-term expression of the cytotoxic gene or because of direct intratumor vector injection. However, because applications of cancer gene therapy are moving toward long-lasting systemic diseases, a safe and efficient delivery method would need a tight control over the levels of therapeutic gene expression (197). One simple way to regulate expression of a transgene is to place it under the control of a promoter that is responsive to a physiological signal, such as glucose elevation or hypoxia (197, 198). Alternatively, regulation of transgene expression may be achieved using exogenous control systems in which gene expression is regulated pharmacologically by administering a small-molecule drug (199, 200). This allows titration into the therapeutic window, dosing to be adjusted as the disease evolves, and therapy to be terminated by drug withdrawal.

Tight regulation of gene expression is a typical feature of the endocrine system. The increasing understanding of tumor biology and the molecular mechanisms involved in gene expression control (*i.e.*, activators, repressors, coregulators) (201) has led to the development of new molecular switches that could be exploited for gene therapy applications and functional genome research.

A. Physiologically regulated gene expression systems

Heat, hypoxia, glucose deprivation, irradiation, and chemotherapeutic agents up-regulate various genes involved in stress responses. Promoters of these genes are attractive for cancer gene therapy because they depend to a large extent on the biology of the tumor or are already induced by various therapeutic modalities. Genes up-regulated in these conditions include multidrug resistance (*MDR-1*), human heat-shock protein (*HSP*), vascular EGF (*VEGF*), irradiation-inducible early growth response (*Egr-1*), and the tissue plasminogen activator (*tpa*) genes.

Hypoxia is a common feature in many solid tumors and plays a significant role in the resistance of cancer to ionizing radiation and cytotoxic chemotherapy. Cellular hypoxia induces a stress response with up-regulation of many genes involved in shifting cellular respiration toward the glycolytic pathway, increasing erythropoiesis and angiogenesis (202). The promoters of the genes that mediate this adaptive response, *i.e.*, phosphoglycerate kinase 1, erythropoietin, and *VEGF*, contain *cis*-acting hypoxia response elements capable of binding hypoxia inducible factor 1 and related proteins (203–205). The feasibility of tumor targeting by using hypoxia response elements promoters to drive therapeutic gene expression has been demonstrated *in vitro* (206, 207) and *in vivo* (208–210).

Irradiation- and chemotherapy-responsive promoter sequences were identified for *tpa* and *Egr-1* genes. Expression of the radiosensitizing cytokine *TNF- α* under the control of the *Egr-1* promoter followed by either radiotherapy (211, 212) or chemotherapy (213) led to synergistic antitumor effects. Engineering of the *CArG* consensus elements derived from *Egr-1* promoter allowed optimization of enhancer sensitivity to low doses of ionizing radiation (214). Chemotherapeutic agents, such as vincristine and doxorubicin, also induce the *MDR-1* gene, which encodes a membrane-effluxing glycoprotein. Effective tumor-targeting has been achieved by combining *MDR-1* promoter-regulated expression of therapeutic genes and chemotherapy (215).

Heat-shock proteins are induced by a variety of stressful environmental conditions, such as heat, irradiation, hypoxia, acidosis, hypoglycemia, and osmotic changes, which are generally present in poorly vascularized tumors. Inducible *HSP* promoters have been used to drive the expression of a variety of therapeutic genes in experimental tumor models after hyperthermia therapy or glucose starvation conditions (216–221), as well as to enhance the oncolytic effect of replicative viruses (222, 223).

B. Pharmacologically regulated gene expression systems

A number of drug-related gene expression systems are available whereby targeted gene transcription is controlled

through the use of small-molecule inducing compounds (101), such as the antibiotics tetracycline (224), streptogramin (225), and macrolides (226); the insect steroid ecdysone or its analogs (227); the antiprogestin mifepristone (RU486) (228–231); and chemical dimerizers represented by the immunosuppressant rapamycin and its analogs (232, 233).

1. Mifepristone-inducible system. At variance with other systems using regulatory proteins of nonhuman origin, the mifepristone system is based on a mutant human progesterone receptor, thus minimizing problems of potential immunogenicity. Like other nuclear receptors of the steroid-hormone superfamily, the progesterone receptor consists of three major functional domains; *i.e.*, the DNA binding domain, the ligand binding domain, and the transactivation domain, which can be interchanged with correspondent elements of other receptors to generate chimeric molecules. The progesterone receptor used in this system has a deletion in the carboxy terminus of the ligand binding domain so that it no longer binds to the agonist progesterone but is still capable of binding to the antagonist mifepristone (234). This mutant is fused to the DNA binding domain of the yeast transcription factor gal4 and the transactivation domain of the HSV VP16 protein, yielding the GL-VP transcription factor (235). Alternatively, the transactivation domain of the chimeric transcription factor may be represented by the activation domain of the nuclear factor- κ B p65 subunit. Moreover, to further reduce the risk of inducing a host immune response, the yeast gal4 DNA binding domain could be replaced by a DNA binding domain of human origin. In the presence of mifepristone, this chimeric regulator binds to genes with upstream gal4 recognition sequences and efficiently activates transcription of the target transgene. The efficiency of the mifepristone-regulated system has been demonstrated by incorporating it in recombinant viral and nonviral vectors (236, 237).

2. Ecdysone-inducible system. This system is based on the use of the insect molting steroid hormone ecdysone and its receptor, the ecdysone receptor (EcR), which is a member of the nuclear receptor superfamily. In the ecdysone-inducible system, a chimeric protein (VgEcR) composed of the VP16 activation domain fused to an EcR with altered DNA-binding specificity heterodimerizes with the retinoid X receptor (RXR) and binds a unique synthetic response element not recognized by natural nuclear hormone receptors. Upon exposure to ecdysone or the synthetic analog muristerone, the VgEcR/RXR complex efficiently induces transgene expression (238, 239) (Fig. 2). Advantages of this system include lower basal activity and higher inducibility compared with other regulated systems and absence of ecdysone effects on mammalian cell physiology. Moreover, ecdysteroids have a lipophilic nature favoring efficient penetration into all tissues including the brain, possess short half-lives that allow for precise and potent inductions, and exhibit favorable pharmacokinetics that prevent storage and expedite clearance (227). This system has been effectively used to generate transgenic mice (227) and inducible viral vectors (240).

3. Glucocorticoid-inducible system. Dexamethasone is also suitable to be used as an inducer, because it can selectively bind

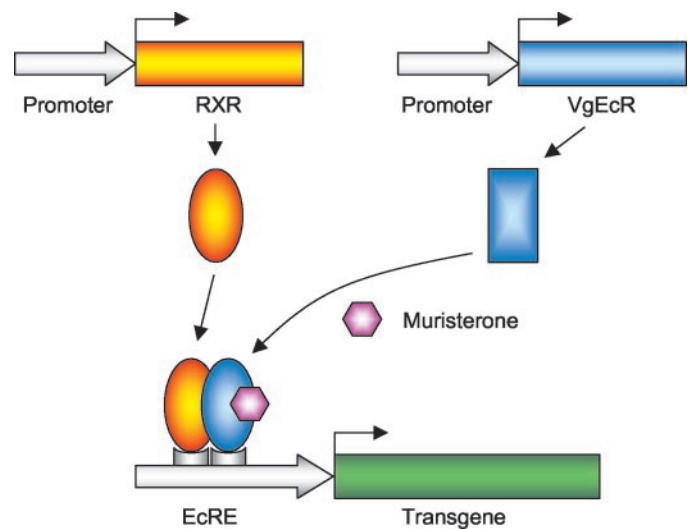


FIG. 2. Schematic diagram of ecdysone-inducible gene expression system. The modified ecdysone receptor (VgEcR) and RXR can heterodimerize in the presence of inducer (muristerone) and transactivate the ecdysone-responsive element (EcRE)-containing promoter.

and activate the p21 promoter in rat hepatoma cells via a glucocorticoid-responsive region. Although this region does not contain a canonical glucocorticoid response element, it can confer specific dexamethasone responsiveness to heterologous promoters (241). A glucocorticoid-inducible retroviral vector was generated by placing the transgene under the control of a minimal synthetic promoter composed of five tandem glucocorticoid response elements upstream to a TATA box. In transduced cells, transgene expression was dexamethasone-inducible and reversible, whereas it was low in the absence of exogenous synthetic corticosteroids (242). A similar construct, containing five tandem repeats of the glucocorticoid-responsive element and the adenovirus major-late promoter, was used to modulate *VEGF* gene expression (243). A glucocorticoid-inducible transcription system, containing reiterated steroid-responsive *cis* elements, was also used to generate a HSV-1 amplicon vector with glucocorticoid-inducible gene expression (244). Glucocorticoid treatment produced up to 50-fold transgene induction in transduced cells.

4. Tamoxifen-inducible system. Disadvantages of transcriptionally regulated inducible systems are represented by basal activity in the absence of induction and low inducibility. As an improved hormone-dependent strategy for regulating protein expression at a posttranslational level, fusion of the hormone-binding domain of the transcriptionally inactive mutant of the murine ER has been adopted (245–248). The modified receptor is unable to bind estrogen yet retains normal affinity for the synthetic ligand 4-hydroxy-tamoxifen (4-OHT). After administration of ligand, the ER fusion proteins are rapidly activated by allowing translocation from the cytosol to the nucleus (249). The effects and pharmacology of nonsteroidal antiestrogens, such as tamoxifen and its derivatives, have been well characterized in animal and human trials, confirming their suitability for gene therapeutic approaches in humans (250). This regulated system was used for the development of an inducible adenoviral vector for

cancer gene therapy. Activity of the *E2F1* gene, encoding a transcription factor that triggers massive apoptosis in several human cancers, was made 4-OHT-dependent by fusion to the ligand binding domain of the ER (251). Upon 4-OHT administration, the ER-E2F1 fusion protein translocated from the cytosol to the nucleus, transactivated E2F-dependent promoters, and rapidly induced cytotoxicity both *in vitro* and *in vivo* (251).

Another approach to designing an ER-based inducible system was the construction of chimeric regulators containing the human ER ligand binding domain and a Cys (2)-His (2)-type zinc finger DNA binding domain. Cys (2)-His (2)-type zinc finger domains are common among human DNA binding proteins and can be engineered to selectively bind different DNA sequences. These chimeric regulators demonstrated a very efficient drug-dependent transgene induction *in vitro* and *in vivo*, after adenovirus-mediated gene delivery to mice (252). Moreover, specific point mutation in the ER ligand-binding domain that ablated estrogen binding enabled selective *in vivo* regulation by tamoxifen (252).

5. Other steroid hormone-inducible systems. Other inducible systems based on the use of steroid hormones include the thyroid hormone-, androgen-, and vitamin D₃-regulated systems. A thyroid hormone-responsive system was developed by using three copies of palindromic thyroid hormone/retinoic acid-responsive element to drive transgene expression. Variations of thyroid hormones and all-*trans*-retinoic acid levels within their physiological range allowed *in vivo* regulation of transgene expression (253). Tissue-specific, thyroid hormone-mediated expression of toxic genes for gene therapy of gliomas was achieved by using the promoter of the myelin basic protein, which contains a thyroid hormone response element (254). Tissue-specific inducible expression of therapeutic genes was also achieved by using a human osteocalcin promoter, which is activated by vitamin D₃, to drive the early adenoviral *E1A* and *E1B* genes. Not only did this promoter allow selective replication of the oncolytic adenovirus in osteocalcin-expressing cells, but it also enhanced viral replication of at least 10-fold upon vitamin D₃ exposure (167).

Androgen-responsive expression of therapeutic genes has been widely used for prostate cancer gene therapy. Androgen response elements have been identified and characterized in the enhancer/promoter region of a variety of androgen-inducible genes, and engineered to attain enhanced transactivation efficiency (154, 255). New inducible systems, based on these naturally evolved switches, might be developed by manipulation of steroid hormone nuclear receptors or their response elements (256, 257).

6. Comment. As shown for gene therapy targeting strategies, endocrinology might greatly contribute to the development of regulated transgene expression systems. Regulated endocrine axes might ideally represent a paradigm of any gene switch systems, and knowledge in the field of endocrinology should be translated in the set-up and refinement of these gene expression tools. Potential applications will range from oncology to cardiovascular diseases, hormone deficiencies, and inherited diseases.

III. Endocrine Cell-Specific Genes as New Therapeutic Tools

Genes expressed in endocrine glands, such as sodium/iodide symporter (*NIS*), noradrenaline transporter (*NAT*), and somatostatin receptors, play a significant role in the diagnosis and treatment of endocrine tumors. Cloning and characterization of these genes have allowed the potential exploitation of their antitumor properties for the treatment of tumors arising from tissues other than endocrine glands.

A. Sodium/iodide symporter

The iodide transporter *NIS* is an intrinsic plasma membrane protein that mediates the active transport of iodide in the thyroid, lactating mammary gland, stomach, and salivary glands (258, 259). The presence of *NIS* in the thyroid gland is exploited in diagnostic scintigraphic imaging and radioiodide therapy of thyroid cancer. The demonstration of *NIS* expression in breast cancer, but not in normal breast tissue (260), suggests the potential use of radioiodine as a diagnostic and therapeutic tool also for nonthyroid cancers in which *NIS* is functionally active. Moreover, ectopic *NIS* expression in cancer cells by gene transfer may be exploited for both diagnostic and therapeutic purposes. In this regard, *NIS*-transduced tumor cells exhibited efficient iodide accumulation, either in culture or in xenografted tumors in nude mice, and were selectively killed by radioiodide (261–264). In a mouse model of intracerebral gliomas, which had been retrovirally transduced with human *NIS*, tumors could be imaged by ^{99m}TcO₄ and ¹²³I scintigraphy and underwent a significant regression after treatment with ¹³¹I (265). Moreover, radioiodide uptake and *NIS* expression in the thyroid gland could be reduced by feeding a T₄-supplemented diet (264), thus preventing thyroid toxicity. Tissue-specific, androgen-dependent iodide uptake has been induced in prostate cancer cells *in vitro* by PSA promoter-directed *NIS* expression (266). Transfected tumors showed a significant regression after single-dose radioiodide therapy in animal models of prostate cancer (267). Adenovirus-mediated *NIS* gene transfer followed by radioiodide administration resulted in highly active iodide uptake and significant tumor volume reduction (268). However, less enthusiastic results have been reported in other tumor models, in which a rapid radioiodide efflux due to lack of iodide organification and intracellular retention was demonstrated (269–272). An amelioration of iodide kinetic was achieved by thyroid ablation and low-iodide diet, although this regimen, in combination with radioiodide therapy, did not inhibit tumor development (273). Cotransfection of non-small-cell lung cancer with both *NIS* and the thyroperoxidase (*TPO*) gene, which catalyzes iodination of proteins and subsequent iodide retention within thyroid cells, resulted in an increase in radioiodide uptake and retention and enhanced tumor cell killing (272). However, by using an adenoviral vector to deliver *NIS* and *TPO*, the levels of iodide organification achieved were too low to significantly increase iodide retention (274).

B. Noradrenaline transporter

Metaiodobenzylguanidine conjugated to ^{131}I -iodide is an effective agent for targeted radiotherapy of tumors of neural crest origin that express the NAT, *i.e.*, pheochromocytoma, neuroblastoma, carcinoid and medullary thyroid carcinoma. Transfer of the NAT gene into nonneuroectodermal tumors would allow targeting of ^{131}I -MIGB to a wide range of tumor types for which no specific and targetable characteristic currently exist. An attractive feature of tumor targeting with ^{131}I -MIGB is that cancer cells that fail to accumulate a lethal quantity of ^{131}I -MIGB may still absorb β -radiation from neighboring targeted cells. The feasibility of this approach was demonstrated by transfecting cell lines with NAT (275–278). In particular, human glioblastoma cell lines transfected with bovine NAT showed a 15- to 25-fold enhancement of radionuclide uptake and dose-dependent cell killing (277, 279). By using cells grown as either monolayer cultures or spheroids, ^{131}I -MIGB was twice as toxic for cells in spheroids compared with cells in monolayers, consistent with a greater radiation cross-fire effect (radiological bystander effect) from ^{131}I β -radiation in the three-dimensional tumor spheroids (277, 280). Moreover, efficient and tumor-selective NAT expression was achieved by placing the transgene under the transcriptional control of the telomerase RNA promoter (281).

C. Somatostatin receptor

Somatostatin and its analogs suppress the growth of tumor cells that express somatostatin receptors, such as neuroendocrine tumors (282). This antiproliferative effect is mediated by somatostatin receptor subtype 1 (*sst1*), *sst2*, and *sst5* (283). Tumor progression is often associated with the loss of differentiated functions, including expression of somatostatin receptors. In this regard, restoration of somatostatin responsiveness in tumor cells by *sst2* gene transfer has been demonstrated to be an effective gene therapy approach for human pancreatic adenocarcinomas (284–286), which, typically, show a specific loss of *sst2* expression (287). Stable transfection of these cells with human *sst2* resulted in the induction of a negative-autocrine loop with secretion of endogenous ligand that activated constitutively the recombinant *sst2* receptor (284, 285, 288). *sst2*-Expressing cells showed significant reduction of cell growth and tumorigenicity both *in vitro* and *in vivo* (284, 285, 288), and this antitumor effect was enhanced by administration of the cytotoxic somatostatin analog AN-238 (288). Moreover, a significant bystander effect and inhibition of metastatic progression was reached when only 25% of tumor cells expressed *sst2* (284, 288, 289).

Aside from antitumor activity, *sst2* gene transfer has been exploited for *in vivo* noninvasive nuclear imaging of tumors. After transduction of tumor cells with viral vectors encoding the *sst2* gene, tumor masses were visualized using radiolabeled somatostatin-avid peptides (290–293). Transduction of tumors with vectors coexpressing a therapeutic gene together with *sst2* allows noninvasive *in vivo* monitoring of the efficacy of gene therapy, as demonstrated in ovarian and lung cancer models in mice injected with bicistronic adenoviruses carrying both *HSV-TK* and *sst-2* (292, 293).

Comment. The idea of noninvasive *in vivo* monitoring of the efficiency of gene therapy by radionuclide imaging has already moved to the clinic, with a protocol of gene therapy for glioblastoma multiforme based on the intratumor delivery of *HSV-TK* followed by radiolabeled nucleoside analog administration (294). Preclinical results in animal models indicate *NIS*, *NAT*, and *sst2* gene therapy could also be effectively applied in humans for diagnostic and therapeutic purposes. Specificity and stability of the interaction between the endocrine transporter, receptor, or enzyme and the ligand or substrate are critical for the success of these therapeutic approaches.

IV. Endocrine Side Effects of Gene Therapy

Endocrine side effects in the course of cancer gene therapy have rarely been assessed in experimental models and in clinical trials. Side effects may include direct cell and tissue injury in endocrine glands, as well as impairment of hormone production. Etiological factors are represented by chemical and biological agents, including nonviral and viral vectors; drugs, such as those used in suicide/prodrug activation therapy; cytokines; and inflammatory and immunological reactions to vector or transgene delivery (Table 4).

A. Nonviral vectors

To the best of our knowledge, no studies on the effects of nonviral gene delivery systems on endocrine glands have been published so far in the literature. However, *in vitro* and *in vivo* evidence of significant cytotoxicity and stimulation of immune response suggests that these effects may also involve the endocrine system.

Nonviral gene delivery (reviewed in Refs. 295–297) refers to the use of naked DNA (298), cationic lipids formulated into liposomes and complexed with DNA (lipoplexes) (299, 300), cationic polymers complexed with DNA (polyplexes) (301), polymeric vesicles complexed with DNA (302), or a combination of both cationic lipids and cationic polymers complexed with DNA (lipopolyplexes) (303). There have also been attempts to combine the benefits of viral and nonviral systems into one delivery vehicle (304).

Cationic liposomes and cationic polypeptides are efficient reagents for the transfer of nucleic acids to cells *in vitro* and *in vivo*. These reagents have several advantages over other methods of nucleic acid transfer; however, toxicity remains a significant problem, especially *in vivo*. These vehicles have been used in several studies, including phase I and II clinical trials (299). In cancer gene therapy, lipoplexes are generally delivered through iv or intraarterial administration, or direct intratumor injection, to limit toxicity to the targeted tissues (reviewed in Ref. 299). One of the drawbacks of intratumor administration is the localization of the delivered nucleic acids predominantly in the needle track. When administered systemically by iv injection, the distribution is mainly in the lung, followed by liver, spleen, and kidney, whereas the intraarterial route allows selective delivery to the target lesion (305–308). In cell culture, lipoplexes cause several changes to cells, including cell shrinking, growth inhibition, and vacuolization of the cytoplasm (309). Cationic lipids may

TABLE 4. Endocrine side effects of vectors used in gene therapy

Vectors	Side effects (Refs.)
Nonviral vectors	
Cationic liposomes and cationic polypeptides	Cytotoxicity, inflammation (295–297)
Viral vectors	
Adenoviral vectors	Inflammation, immune response (9) Selective adrenocortical toxicity after systemic delivery, with degeneration and hemorrhage in the zona reticularis, and impairment of steroidogenesis (9, 342) Inflammation and focal necrosis after intrapituitary injection (348)
AAV vectors	Inflammation, immune response (380–382)
HSV vectors	Inflammation, immune response (394, 396) Impairment of pituitary hormone production (397)
Retroviral vector	Inflammatory response to vector-producing cells (36, 82, 84, 88, 410, 411) Impairment of hormone production by pituitary and adrenal cells (421) Autoimmunity (428–430) Oncogenesis (16–20) Germ line transmission? (591, 592)
Lentiviral vector	Interaction between viral sequences or proteins and steroid hormone receptors (443) Oncogenesis? Germ line transmission?

?, Hypothesized.

TABLE 5. Oncolytic viral infection of endocrine cells

Oncolytic virus	Effects
Adenovirus	Thyroid: no toxicity of <i>dI1520</i> in normal rat thyroid cells (500); transformation of thyroid cells (501, 502)? Adrenal: selective adrenotropism of adenoviruses (335, 336, 503, 504); focal necrosis in all cortical zonae (335, 336, 342, 503, 504) Pituitary: inflammation, necrosis?
HSV	Adrenal: adrenal tropism; necrosis and hemorrhage (516–519, 525–532) Pituitary: activation of the HPA axis; impairment of hormone production (398, 523)
Reovirus	Adrenal: adrenalitis and marked cytopathic effect (542, 543) Pituitary: cytopathic effect (544, 545), GH deficiency (545) Endocrine pancreas: destruction of β -cells (545–549) Thyroid: thyroiditis (553–557) Autoimmunity (pancreas, anterior pituitary, thymus, thyroid, gastric mucosa) (540, 541)
Newcastle disease virus	ACTH and cortisol hypersecretion (578–580)

?, Hypothesized.

also induce hemolysis (310) or fusion between erythrocytes (311). *In vivo* studies demonstrated toxic effects mainly in the injection site, such as inflammation of the eyes after intraocular instillation (312), epileptic seizures, and, in severe cases, death after intracerebral injection in mice (313, 314), inflammatory response after intraarticular delivery (315), epithelial cell death after intratracheal administration (316), and complement activation via the alternative pathway (317). Cationic liposomes have also been reported to induce acute systemic inflammatory reactions (299, 318) and macrophage and neutrophil infiltration into the lungs of mice when administered intratracheally (319). Nephrotoxicity (320) and hepatotoxicity (321) were reported after delivery of lipoplexes via the renal artery and the portal vein, respectively. Intravascular delivery of lipoplexes may lead to embolization and microinfarctions, as demonstrated after iv, intraarterial, or intracoronary administration (322). These complexes were also found to be highly toxic when administered orally, provoking a dramatic hypothermia resulting in death in some mice (323). Systemic administration of lipoplexes stimulates the production of proinflammatory cytokines, such as

TNF- α , IFN- γ , IL-6, and IL-12 (318, 319, 324, 325). This effect is mainly related to the plasmid DNA component of these vectors and the cytosine-phosphate-guanine motifs contained within (326). Although potentially associated with significant side effects, these immunostimulatory properties may be exploited for cancer immunotherapy. Effects on the endocrine system of cytokines, including TNF- α , IFN- γ , IL-6, and IL-12, will be discussed in *Section IV.C*.

Cationic polymers, such as polylysine, histones, and dendrimers, are able to interact electrostatically with the DNA molecule and condense into compact particles (polyplexes). Condensation prevents DNA degradation by nucleases and allows internalization of particles into cells by natural processes such as endocytosis, pinocytosis, and phagocytosis. To improve transfection efficiency, cell-binding ligands have been incorporated into these transfection vehicles, resulting in receptor-mediated mechanisms for cellular uptake. Positively charged polycation/DNA complexes were found to aggregate at physiological salt concentrations, to interact with components of the coagulation and complement system, and to cause aggregation of erythrocytes that can result

in microembolism (301, 303, 327–329). This process may be enhanced by the avid binding of the positively charged complexes to cell membranes (330–332). Nonspecific interactions with plasma components or erythrocytes can be prevented by shielding the surface of transfection particles with hydrophilic molecules, such as polyethylene glycol (329) or transferrin (333, 334). Systemic delivery of nonshielded complexes into tumor-bearing mice resulted in high transgene expression in the lungs and lower gene expression in other organs, such as heart and liver, but was often associated with severe toxicity (328), particularly when high molecular weight complexes were used (303). At variance, shielded complexes preferentially accumulated in tumor tissues (297, 299, 333, 334).

B. Viral vectors

Endocrine side effects in the course of cancer gene therapy could be due to untoward infection of endocrine cells by nonreplicative viral vectors, active viral replication and lysis of endocrine cells in the case of replicating viruses, and toxic effects due to stimulation of cytokine production by these vectors. Endocrine side effects caused by replication-deficient viral vectors will be discussed in this section, whereas infection of endocrine cells by wild-type and oncolytic viruses will be dissected in *Section V*.

1. Adenoviral vectors. Among endocrine glands, adenoviral vectors have a natural tropism for adrenocortical cells, as demonstrated in animal models and in humans (9, 335–342). One of the major factors in determining adrenal targeting after intravascular delivery is the presence of fenestrations in the microcirculation of the adrenal gland, which may allow a direct contact of the vector with adrenal cells. The lack of delivery to most other parenchymal tissues is most probably due to the endothelium, which acts as an anatomical barrier. Endothelial cells are relatively resistant to adenoviral infection, because of low levels of expression of the coxsackie-adenovirus receptor (CAR) (343, 344). Indeed, there is little correlation of the tissue distribution of CAR or integrins with expression from adenoviral vectors (343).

Intravenous administration in a baboon of high doses of a first-generation nonreplicating human adenoviral vector expressing β -galactosidase resulted in fatal toxicity, with thrombocytopenia, increased liver enzymes, and severe endothelial injury (9). At necropsy, there were pleural and pericardial effusions; hemorrhages in lymph nodes, testes, and spleen; and brain congestion. Histological examination demonstrated the presence of the vector (*i.e.*, expression of β -galactosidase) in the liver, red pulp of the spleen, vascular system, the lymph node sinus, and rare acinar pancreatic cells. Adrenocortical cells frequently showed expression of vector, whereas the parenchyma of other organs was negative. In particular, neither germ cells and Sertoli cells nor interstitial cells in the testis were positive. Injury to the vascular endothelium was the most prominent histological abnormality. Focal degeneration was present in the adrenal, particularly in the zona reticularis with congestion and hemorrhage as well as a few neutrophils. The periadrenal tissues showed vascular dilatations and increased numbers of in-

travascular leukocytes. No abnormalities were identified in other endocrine glands including pancreas, testis, thyroid, and parathyroid. A 10-fold lower dose of the vector did not cause any significant toxicity or changes in laboratory values in another injected baboon (9). At necropsy, the microscopic abnormalities consisted of enlargement of spleen and lymph nodes, but no other abnormalities were identified. Expression of β -galactosidase was detected in liver, spleen, pancreas, and adrenal, but not in other tissue parenchyma. Positively staining adrenocortical cells were few to moderate in number. No histological abnormalities were observed. Increased levels of IL-6 and TNF- α , but not IL-8, were observed only in the baboon injected with the higher adenoviral vector dose (9). Immunocompetent mice administered with three doses of the vector (*i.e.*, half the dose, equivalent to the dose, or twice the dose administered to the high-dose baboon) showed similar hepatotoxicity and endothelial injury at the higher vector doses (9).

Adenoviral tropism for the adrenal gland was also demonstrated in animal studies of fetal gene therapy. Administration of a nonreplicating human adenoviral vector to guinea pig fetuses in the late stage of pregnancy through the umbilical vein led to prominent transgene expression in liver and moderate expression in spleen, adrenal gland, and heart (339). In a mouse model, the pattern of gene expression was dependent on the developmental stage of the embryo at the time of virus administration (338). The most extensive gene transduction was detected in embryos injected at 15 d. When injected at this embryonic stage, endothelial cells of the adrenal expressed the transgene besides other organs, whereas no adrenal transduction was detected when embryos were injected at other stages (338).

In vitro infection of primary cultures of isolated bovine adrenocortical cells by nonreplicating first-generation human adenoviral vector type 5 demonstrated that cell transduction was followed by specific ultrastructural alterations (342). The most significant changes involved mitochondria, which appeared pleiomorphic, exhibiting a reduced amount of tubular internal membranes. The cristae had a lamellar morphology, characteristic of nonsteroidogenic cells or steroidogenic cells after deprivation of trophic hormones, and the mitochondria matrix contained crystalline structures. Segmentation of the nucleus and the presence of intranuclear viral particles were also observed (342). Adrenocortical cell proliferation was increased after transduction. Adrenocortical response to ACTH was significantly suppressed by vector infection, whereas basal steroidogenesis was increased, probably related to the increased cell proliferation (342). Similar modifications in patterns of steroidogenesis have been reported in Y-1 mouse adrenal tumor cells after transformation by a simian adenovirus (345, 346).

Data in the literature on side effects caused by adenoviral transduction of other endocrine glands are scarce. Recombinant adenoviral vectors can infect all pituitary cell types and hypothalamic cells effectively, as demonstrated in cell cultures and in animal models stereotactically injected with vector particles (130–136, 347). Anterior pituitary glands injected with recombinant human adenoviral vector type 5 displayed variable degrees of inflammatory response, with periglandular fibrosis, lymphocytic infiltrate, venulitis, and

focal necrosis and/or apoptosis in almost all cases (348). However, adenoviral-mediated transduction of hypothalamus and pituitary cells *in vivo* after systemic delivery seems to be unlikely. Efficient gene transfer to the pituitary and part of the hypothalamus near the pituitary was only achieved when recombinant adenoviral vectors were injected into the amniotic cavity of rat embryos at embryonic d 12, but not at other embryonic periods. Transgene expression persisted at least 1 wk after birth (349). It is conceivable that transduction of the hypophysis occurred through the pharynx, because the adenohypophysis is originally developed from a pharynx segment, and the duct connecting the adenohypophysis to the pharynx is still open at embryonic d 12. The risk of untoward pituitary cell transduction during systemic or intracerebral delivery of recombinant adenoviruses for cancer therapy has not been assessed so far. Moreover, effects on the hypothalamus and pituitary gland of the inflammatory response elicited by vector delivery into the brain have not been investigated. This issue is of interest, because gene transfer into the brain using adenoviral vectors has been demonstrated to induce acute inflammatory reactions (350, 351), whereas peripheral readministration of viral vectors induces a delayed-type hypersensitivity reaction accompanied by localized demyelination, which eliminates transgene expression (350). Examination of the long-term outcome of adenovirus-mediated HSV-TK followed by GCV in a rat syngeneic glioblastoma model demonstrated the presence of active brain inflammation 3 months after successful inhibition of tumor growth (352). The inflammatory infiltrate consisted of activated macrophages/microglia and astrocytes and T lymphocytes and was associated with demyelination. Strong and widespread HSV-TK immunoreactivity and vector genomes were detected throughout large areas of the brain (352). Thus, besides the risk of infection of pituitary and hypothalamic cells, potential endocrine side effects due to persistent transgene expression and long-term cytokine production by inflammatory cells should be evaluated in clinical gene therapy trials for brain tumors. Effects of cytokines in the endocrine system, which have been extensively reviewed in the literature (353–357), are addressed in Sections VI.C. and VI.

Although thyroid cell transduction seems to be an unlikely event after systemic administration of recombinant adenoviral vectors (9), adenoviral proteins could have some effects on thyroid function. Indeed, the adenovirus *E1A* gene products have been demonstrated to interact directly with nuclear receptors, including retinoic acid receptor β (RAR β) (358, 359) and thyroid hormone receptor (TR) (360), and thereby function as a potent cofactor for transcriptional activation. The biological significance of these interactions may be related to the role of RAR β and TR in transcriptional activation of specific genes involved in cell differentiation.

Efficient gene transfer to pancreatic islets *in vitro* and in grafted animals using first-generation adenoviral vectors has been reported (361–367). Gene transfer was not associated with islet toxicity, as determined by measuring glucose-stimulated insulin release (365, 366).

With the exception of cancer, in which an immune response to vector could result in antitumor activity, preexisting immune response is a major cause of inefficiency and

side effects of the commonly used first-generation human adenoviral vectors type 2 or 5 (368). To approach this problem, the use of the so-called “gutless” adenoviral vectors, which are devoid of all sequences encoding for structural proteins, has been suggested (369, 370). As an alternative, recombinant adenoviral vectors of nonhuman origin (*e.g.*, ovine, canine, bovine, porcine adenoviral vectors) could be effectively used to avoid inflammation and immune response (371–376). Some improvement in transgene persistence can also be achieved by repeated administration with human adenoviral vector of different serotypes (*e.g.*, adenovirus type 34), although there appears to be some T cell cross-reactivity in recognizing different human adenovirus subgroups (377, 378).

2. *AAV vectors.* AAVs are human parvoviruses that normally require a helper virus, such as adenovirus, to mediate a productive infection. Lack of pathogenicity and the tendency to establish latent infection through integration into the chromosomal DNA make this virus an attractive vector for gene therapy (379, 380). Because the AAV vector genome lacks viral coding sequences, the vector itself has not been associated with toxicity or any inflammatory response, except for the generation of neutralizing antibodies that may limit re-administration (380–384). The vector can efficiently transduce both dividing and nondividing cells and can be delivered to many different organs, including the central nervous system, liver, lung, and muscle, by *in vivo* administration (379). Several preclinical and clinical studies conducted with AAV demonstrate the efficacy and safety of this delivery system (380, 385–387), which however will best suit application for local gene transfer to target tissues, *e.g.*, brain and skeletal muscle. After local injection, the vector does not spread to surrounding tissues; thus, the risk of untoward infection of nontarget cells, including endocrine cells, should be very limited. On the other hand, efficient *in vitro* transduction of hypothalamic, medullary thyroid, and pancreatic β -cells after direct exposure to the virus has been demonstrated (388–391).

3. *HSV vectors.* Development of highly defective nonreplicative HSV vectors allowed a marked reduction of vector toxicity even for primary neurons in culture, readily killed by less defective HSV vectors (392). Highly defective nonreplicative HSV vectors are most suited for transgene expression in the nervous system in which the virus has evolved to remain lifelong in a latent state (393, 394). Deletion of multiple immediate early genes reduces the cytotoxicity of HSV-based vectors. These vectors are suitable for both short-term applications and long-term gene expression. In the first case, immediate early promoters or exogenous promoters are used to produce high level transgene expression for up to 1 wk after injection. In the latter case, highly defective HSV vectors are used for expression of genes in the nervous system, where they are able to efficiently establish latency in neurons and serve as a platform for long-term gene expression driven by the latency promoter system (395, 396). These mutants cannot reactivate from latency and cannot spread to other nerves or tissues after cell infection. Because most applications involve the nervous system, endocrine cells that could be potentially

involved in toxic effects are hypothalamic or pituitary cells. Indeed, efficient gene transfer after HSV infection has been demonstrated in these cell types (397–399). Experiments performed in primary cultures of normal and hyperplastic rat pituitary cells and in grafted rat pituitaries *in vivo* showed a significant reduction of hormone production after exposure to the vector (398).

4. Retroviral vectors. Retroviral vectors, especially those derived from the Moloney murine leukemia virus (Mo-MLV), are currently the most common type of vector used in gene therapy clinical trials. Properties of these vectors are infectivity over a wide range of cell types, lack of pathogenicity, and integration into the genome of the host cell. Disadvantages include the risk of insertional mutagenesis or activation of oncogenes as a result of random integration of the viral genome into the host cell chromosomes, transcriptional silencing of the integrated transgene, low vector titer, and inactivation by human complement (400–402). The ability of vectors derived from the oncovirus group to selectively transduce cancer cells (which are usually more proliferative than normal cells) is an advantage of this type of retroviral vector for use in cancer treatment. Hence, these vectors have been investigated in clinical trials for the treatment of several cancers including ovarian, breast, brain, and lung cancers. Phase I clinical trials, using retroviral vectors for gene delivery, have demonstrated no toxicity, which is a major advantage of this type of vector over other viral vectors (28, 35, 80, 85, 403–409). To obviate low vector titer and rapid elimination by human complement, retroviral vector-producing cells may be directly injected into the target lesion. In this case, side effects related to the injection procedure or inflammation due to the delivery of xenogenic cells may be observed (36, 80, 82, 84, 88, 410, 411). Moreover, transient detection of vector sequences in peripheral blood leukocytes has been reported in patients who underwent intracerebral administration of retroviral vector-producing cells for gene therapy of brain tumors (36, 82, 84, 86, 88, 411). Immune response to vector or vector-producing cells has been reported in 9% and 50% of treated patients, respectively (411).

Development of a second malignancy as a consequence of insertional mutagenesis has not been demonstrated so far in cancer patients treated with gene therapy. This potential risk should always be weighted with the benefits of gene therapy in this category of patients, especially after the French trial (15–20). It is, however, conceivable that the carcinogenic risk of gene therapy is not higher than that of other cancer treatment regimens, such as chemotherapy and radiotherapy.

Although never demonstrated in patients treated with retroviral vectors or retroviral vector-producing cells, generation of replication-competent virus particles represents an important safety issue. To minimize the risk of replication-competent virus particle production and that of pseudotype formation, improved packaging cell lines of different animal origin have been established. Moreover, vectors have been more carefully designed to contain hybrid LTR and chimeric *cis*-acting sequences and separated in different plasmids for expression of the different retroviral functions. Appropriate deletions in the LTRs have led to the so-called self-inacti-

vating vectors, thus diminishing the risk of insertional mutagenesis (412).

With regard to the endocrine system, normal resting endocrine cells should be rather refractory to retroviral vector transduction because of their low mitotic activity. However, transduction of endocrine cell lines and primary cell cultures by retroviral vectors has been demonstrated (362, 412–414). Retrovirus infection of endocrine glands has been associated with the development of endocrine disorders both experimentally (415, 416) and clinically (417–420). Infection of the murine pituitary cell line GH3 and the murine adrenocortical cell line Y-1 *in vitro* by Mo-MLV resulted in increased cell proliferation without significant cytopathic effect (421). The establishment of active and productive infection in Y-1 cells *in vitro* led to a transient increase in steroid secretion; however, during the continuous passage of infected cells, steroid production was reduced. In contrast, secretion of PRL and GH was markedly reduced in infected GH3 cells during initial and continuous cell passaging. In addition, the reduction in hormone secretion in pituitary cells corresponded with an increase in virus yield. Because the decrease in hormone secretion during infection occurred with no reduction in cell viability, a modulatory effect of Mo-MLV infection on hormone secretion and synthesis was suggested (421).

An intriguing issue is the detection of elevated levels of human endogenous retrovirus expression in endocrine glands (422–425), especially in the fetal adrenal cortex (426). Moreover, the human endogenous retrovirus HERV-R has been shown to be expressed in all layers of the human adrenal cortex, but not in the medulla. High levels of expression were demonstrated also in adrenocortical adenomas, whereas expression was low in pheochromocytomas (427). At variance, preferential expression of the human endogenous retrovirus HERV-E was revealed in the pancreas and thyroid gland (425). These findings suggest a role of endogenous retroviruses in development, differentiation, and/or hormone production by endocrine cells. Endogenous retroviruses have also been related to the pathogenesis of organ- and non-organ-specific autoimmune disorders (428, 429), although this hypothesis still remains quite controversial with regard to endocrine autoimmune diseases (430–432).

Thus, although retroviral vectors should be considered among the safest gene transfer tools, safety issues on the risk of germ line transmission, development of autoimmunity, or malignant transformation remain to be thoroughly elucidated.

5. Lentiviral vectors. At variance with oncoretroviruses, lentiviruses such as HIV type 1 (HIV-1) can replicate in non-mitotic cells. In fact, their preintegration complex, a macromolecular structure that includes the viral genome, a few structural proteins, reverse transcriptase, and integrase can enter into the nucleus without requiring nuclear fragmentation (433). Lentiviral vector particles are generated by co-expressing the virion packaging elements and the vector genome in producer cells. To improve the safety profile of lentiviral vectors, multiple attenuated packaging systems have been created, the latest (“third”) generation of which comprise only three of the nine genes of HIV-1 to prevent reconstitution of the parental virus (434). As done with oncoretroviral vectors, engineering of self-inactivating HIV-1-

derived vectors makes it possible to minimize the risk of emergence of replication-competent recombinants and avoids problems related to promoter interference (435, 436).

Experience with lentiviral vectors from clinical trials is still very limited; however, progress accomplished in the design of such vectors suggests that their biosafety characteristics should be superimposable to those of retroviral vectors. On the other hand, it is conceivable that undesired transduction of slowly replicating endocrine cells by lentiviral vectors in the course of cancer gene therapy should be more frequent than when using oncoretroviral vectors (437–439).

Endocrine dysfunctions observed in HIV-1-infected patients are generally related to opportunistic infection and malignancy occurring in late stages of the disease or to antiretroviral drugs and medications used in the treatment of opportunistic infections, but not to HIV infection *per se* (440–442). It is therefore conceivable that lentiviral vector delivery should not be accompanied by specific side effects on the endocrine system.

On the other hand, hormones may modulate lentiviral vector function. Steroid hormone receptors, including glucocorticoid receptor (GR), TR, RXR, RAR, peroxisome proliferator-activated receptors, orphan receptors, and numerous other host factors have been shown to participate in the regulation of the HIV-1 LTR promoter, and thus in the expression of viral genes (443). Among hormone receptors, GR has been demonstrated to interact with glucocorticoid response elements in HIV-1 LTR (444, 445). Treatment with glucocorticoids led to increased viral production in culture and enhancement of HIV gene expression (445). The HIV-1 virion-associated accessory protein, which functions as a regulator of cellular processes linked to HIV life cycle, has been demonstrated to interact directly with the GR and general transcription factors, such as p300/CBP coactivators, thus acting as an adapter linking transcription components and coactivators (446, 447). Heterodimers of TR/RXR have been shown to bind the critical region of the promoter that contains the nuclear factor- κ B and Sp1 binding sites (448–451) and to repress LTR in the context of chromatin (451). Addition of either T₃ or a histone deacetylase inhibitor relieves this repression, suggesting that TR/RXR heterodimer activates the HIV-1 LTR in the presence of T₃ (449–451) and that transcriptional regulation of chromatinized LTR involves histone acetylation (451).

C. Ectopic expression of cytokines as therapeutic genes

Many of the cytokines that are used in cancer gene therapy as immunomodulating genes exert important effects on the endocrine system. These cytokines are also released in response to immune/inflammatory insults, including viral vector administration. Therefore, gene transfer is expected to trigger the activation of the immune and neuroendocrine systems and the release of a cascade of cytokines with pleiotropic effects on endocrine cells. On the other hand, hormones modulate the effects of cytokine genes on target cells (353).

Among cytokine genes, the most commonly used as therapeutic tools in cancer gene therapy are those encoding for

IL-2, IL-4, IL-7, IL-12, IFN- α , IFN- β , IFN- γ , TNF- α , TGF- β , and GM-CSF.

Effects of cytokines on the endocrine systems have been extensively reviewed elsewhere (353–357); therefore, only a brief summary on this issue will be presented here. IFNs, which are typically produced as a defense against viral infection, were among the first cytokines to be demonstrated to have neuroendocrine effects, *i.e.*, stimulation of steroidogenesis. Activation of the hypothalamus-pituitary-adrenal (HPA) axis was demonstrated also for IL-1, IL-2, or IL-6, IFN- β , IFN- γ , leukemia inhibitory factor, and TNF- α in both animal models and humans (452–454). IL-1, IL-2, IL-6, and TNF- α directly stimulate cortisol secretion by adrenal cells in culture, and IL-1 and IL-6 stimulate pituitary cells to produce ACTH and β -endorphin via stimulation of hypothalamic CRH secretion (454). IL-2, which is widely used in cancer gene therapy as an immunostimulating gene, is more potent than CRH in stimulating ACTH secretion and is the most potent secretagogue for ACTH currently identified (455). This effect, which has been confirmed in cancer patients receiving IL-2 administration (456), might also be observed in the case of systemic IL-2 gene therapy. Among other cytokines used for cancer gene therapy, GM-CSF stimulates ACTH and corticosterone production (457) and melatonin release by the pineal gland (458), whereas IFN- γ , besides stimulating melatonin secretion from pinealocytes (459), up-regulates GR expression by macrophages (460). Induction of melatonin release represents a positive feedback loop, because melatonin itself enhances IFN- γ production (461). TGF- β exerts an inhibitory effect on steroidogenesis (462) and seems to be involved in fetal (463, 464) and adult (465) steroidogenesis, where it seems to reduce the synthesis of dehydroepiandrosterone sulfate.

Regarding the effects of cytokines on other neuroendocrine mechanisms, IL-1, TNF- α , IFN- γ , and IL-6 exert an inhibitory effect on the hypothalamus-pituitary-thyroid axis (466–469), whereas IL-1 inhibits the hypothalamus-pituitary-gonadal axis by decreasing LHRH and LH concentrations (470). Cytokines may also affect sexual steroid production by direct modulation of hormone release from the gonads (355).

Immune cytokines also influence complex mechanisms involving thermoregulation, food intake, sleeping patterns, and behavior (355). Moreover, cytokines induce several metabolic alterations, including increase of insulin levels after IL-1 administration (355).

D. Inhibition of hormones and growth factors

Endocrine therapy has become a standard of care for endocrine-related tumors, including breast, ovarian, and prostate carcinoma, and has brought a significant improvement of patients' outcome. However, systemic treatment is often poorly tolerated and associated with long-term side effects (471–476). Gene therapy approaches, allowing local delivery of therapeutic genes or selective inhibition of protein expression by antisense oligonucleotides or ribozymes, should represent an advantage over conventional drugs in terms of toxicity and efficacy. So far envisaged gene therapy strategies based on the inhibition of hormones or growth

factors include the use of antisense oligonucleotides or ribozymes directed against androgen receptor (477–480), ER (481), and various growth factors or their cognate receptors, such as VEGF (482), EGF (483–486), and IGFs (487–496). Some of these approaches have already entered clinical trials without unusual side effects (1, 2, 72). While further data are being achieved from experimental studies and ongoing clinical trials, a careful investigation on the potential impairment of endocrine physiology should be carried out.

Comment. Side effects of cancer gene therapy on the endocrine system have rarely been assessed so far both in pre-clinical studies and in clinical trials. They are, however, unlikely to occur with nonreplicating viral vectors and, if present, are generally related to an acute inflammatory reaction after vector or xenoantigen delivery. Because gene therapy of cancer is moving toward more ambitious purposes, *i.e.*, long-term cure of diseases, careful evaluation of safety issues and long-term outcome of treated patients should be of primary importance.

V. Oncolytic Virus Infection of Endocrine Cells

The idea of using viruses as oncolytic agents originated at the beginning of the 20th century, when spontaneous tumor regression was observed in some patients with viral infection or after rabies vaccination (497). This idea was temporarily abandoned after clinical experience with a variety of wild-type viruses, due to low efficacy and significant toxicity (497, 498). The field of viral therapy for cancer has been reborn in the last decade, with the development of viruses engineered to replicate selectively in tumor cells, and several clinical trials with oncolytic viruses are currently ongoing.

Since there is a total lack of information on the effects of replication-selective viruses on the endocrine system, indirect assumptions could be obtained from wild-type viruses. Even with important limitations (oncolytic viruses are engineered to selectively replicate in cancer cells and thus are generally attenuated), wild-type viruses might represent a model for oncolytic viral infection and thus could provide important information for vector design and safety evaluation.

A. Oncolytic adenoviruses

Replication-competent oncolytic adenoviral vectors are generated by deletion of gene functions that are critical for viral replication in normal cells but not in tumor cells (reviewed in Refs. 497–499). In particular, deletion of the *E1A* or the *E1B* gene, whose products inactivate the tumor suppressor proteins pRB and p53, respectively, allows adenoviral replication in pRB- or p53-defective tumor cells, but not in normal cells with intact pRB and p53 pathways. Another strategy to achieve tumor-selective adenoviral replication is the use of tumor- or tissue-specific promoters to drive the expression of an adenoviral gene that is critical for efficient viral replication, such as *E1A* (497, 498). Several studies demonstrate that tumor selectivity is not absolute, and efficient replication may also occur in nontarget cells (499). Thus, potential cytotoxic effects, after systemic administra-

tion of replication-competent adenoviral vectors, should be considered.

1. *Thyroid gland.* Efficient infection of the thyroid gland by adenoviral vectors has been well documented in the literature (104). With regard to oncolytic adenoviral vectors, infection with the *E1B* 55-kDa gene-defective adenovirus *dl1520* efficiently induced cell death in p53-defective thyroid carcinoma cell lines and reduced growth of xenografted thyroid tumors in nude mice (500). Conversely, the virus did not produce cytopathic effects on normal rat differentiated cells (501), suggesting that it should not be toxic for thyroid cells if systemically delivered. On the other hand, a phase I clinical study based on iv infusion of the *dl1520* adenovirus in patients with metastatic cancers bearing p53 mutations, including a patient with papillary thyroid carcinoma, showed evidence of viral replication and cytokine response (54). In particular, most patients, including the patient with thyroid cancer, experienced transient fever, nausea, and fatigue after virus administration. Disease stabilization was observed in all but one case that progressed (54).

Effects of adenovirus on thyroid cell differentiation and transformation should also be taken into account. Transfection of the adenovirus *E1A* gene into a rat-differentiated thyroid cell line induced a block in the expression of differentiated functions, although without the appearance of typical transformation markers (501). Indeed, *E1A*-transformed thyroid cells maintained sensitivity to growth inhibition induced by cAMP (502). A highly malignant phenotype was achieved by cooperation of adenovirus *E1A* with other oncogenes (501).

2. *Adrenal gland.* As reported above, several studies demonstrated that the adrenal gland is a major target for adenoviral infection. In particular, selective adrenotropism of adenovirus has been demonstrated in mice (335, 336) and calves (503, 504) during experimentally induced infections. In the experimental mice model, adenoviral infection involved all three zones of adrenal cortex, with 80% or more of the cells exhibiting intranuclear inclusions (335) associated with virion accumulation (336). In calves, acute focal nonsuppurative necrosis was present in the zona glomerulosa and fasciculata and occasionally in the zona reticularis and medulla of the adrenal glands, with pyknotic nuclei and eosinophilic intranuclear inclusions (503, 504). Nonspecific histological changes have also been observed in infants with generalized adenoviral infections, including edema of the capsule and stroma, loss of trabecular structure, delipoidization in the definitive adrenal cortex, and reabsorption of the elements of the fetal cortex (337).

The *E1B*-deleted *dl1520* oncolytic adenoviral vector was used for the treatment of a patient with metastatic adrenocortical carcinoma, resulting in disease stabilization. The patient, who had transient fever after iv virus administration, was taken off the study due to renal failure after 103 d of follow-up (54).

The effect of replication-competent adenoviral vectors in adrenocortical cells was analyzed in primary cultures of bovine adrenal cortical cells transduced with different adenoviral deletion mutants. Whereas *E3/E4*-deleted vectors did

not affect adrenocortical morphology and function, including steroid secretion, nonreplicative E1/E3-deleted viruses led to alteration of the ultrastructure and increased proliferation of adrenocortical cells, associated with suppression of response to ACTH stimulation (342). These findings suggest a role for adenovirus E4 region in the impairment of steroid secretion.

3. *Pituitary gland.* Although infection of pituitary cells with replication-competent oncolytic adenoviral vectors has not been assessed so far in the literature, efficient transduction of all pituitary cell types by nonreplicative recombinant adenoviral vectors has been demonstrated in several *in vitro* and *in vivo* studies (130, 133, 505–507). The evidence of a severe inflammatory reaction, with periglandular fibrosis, lymphocytic infiltrates, venulitis, and focal necrosis in ovine pituitary glands injected with first-generation adenoviral vectors (348) indicates the need for evaluation of possible adverse effects in the normal pituitary gland after adenoviral vector-mediated gene delivery into the central nervous system.

B. *Oncolytic herpes simplex viruses*

As with the oncolytic adenoviruses, two strategies are used to target HSV-1 replication to tumor cells. The first involves deletion or inactivation of viral genes that are essential for viral replication in normal cells but dispensable in tumor cells, such as HSV-TK, ribonucleotide reductase, and γ 34.5 (497, 508). The second is based on the use of cellular tumor-specific or tissue-specific promoters to drive the expression of a viral gene that is critical for viral replication, such as the immediate-early *ICP4* gene (509).

Data in the literature on infection of endocrine glands with replicating herpesviruses are scarce. However, reports of adrenal infection by HSV-1 and HSV-2 both in humans (510, 511) and in experimental models (512–518) and documented effects of HSV-1 on the hypothalamo-pituitary-adrenocortical axis (519, 520) indicate that systemic or intracerebral delivery of replicative HSV-based vectors could be potentially associated with life-threatening adverse events. Although mutations in critical viral sequences markedly decrease neurovirulence and cytotoxicity of oncolytic HSV, distant indirect effects, such as activation of the HPA axis could persist. Other safety concerns in the case of HSV vector inoculation into the tumor or into the brain are virus leakage into the bloodstream or infection of susceptible distant organs. Moreover, vector inoculation could lead to reactivation of, complementation, or recombination with a patient's latent virus to cause disease. Although a rare event (521), it has been shown in mice that mixed infections with two nonlethal nonneuroinvasive HSV-1 strains can result in a lethal infection and spread to the brain (522).

1. *Pituitary gland.* HSV-based vectors are capable of transducing pituitary cell lines and normal and hyperplastic anterior pituitary cells *in vitro* (397) as well as *in vivo* in ectopic grafts of normal anterior pituitary glands and in estrogen-induced prolactinomas in rats (523). Infection of normal and hyperplastic anterior pituitary cells leads to a significant reduction of hormone release *in vitro* and in pituitary-grafted animals *in vivo* (398). Replication of oncolytic HSV vectors

in pituitary or hypothalamic cells has not been investigated so far.

2. *Adrenal gland and other endocrine glands.* Clinical cases of involvement of the adrenal gland in the course of disseminated wild-type HSV infection, with the presence of hemorrhagic necrosis in the adrenal cortex and evidence of HSV particles, have been reported in the literature (510, 511, 524). The adrenal gland is a major target of HSV-1 infection. The propensity of HSV to localize in the adrenals was demonstrated after several routes of administration in animal models, including intranasal (525), sc (526), iv (517, 527, 528), ip (529–531), vaginal (516, 532), and intraocular injection (515). Experimental intranasal infection of mice with wild-type HSV-1, VR3 strain, was constantly followed by interstitial pneumonia and adrenal necrosis, often leading to death (525). Viral antigens were detected in the lungs and the adrenals 24–48 h after inoculation, suggesting that the virus reached the adrenal gland via hematogenous dissemination, possibly initiated by virus invasion of the pulmonary vessels. At variance, no histological lesions were detected in the thyroid gland and pancreas (525). Extensive adrenocortical necrosis, beginning in the zona fasciculata and subsequently involving the other zonae, was a constant finding along with the presence of the virus in the affected adrenal glands as confirmed by immunohistochemistry (525). Subcapsular cell proliferation developed in the damaged adrenal cortex as early as 12 d after inoculation with HSV-1, a finding particularly evident in mice surviving up to 1 yr after virus inoculation. Adrenals from these latter animals still released infectious HSV when cocultivated with susceptible cells (525).

With regard to the iv route of infection, replication of HSV-1 in the adrenal gland of mice was observed 12 h after inoculation, peaked at 48 h, and was maintained until death (517). In particular, infection involved the zona fasciculata and induced cell apoptosis. Lesions involved the medulla 48 h after inoculation. In the adrenal medulla, cells were fused and formed multinucleated giant cells but rarely displayed clear signs of cell death. Macrophages, which serve as a frontal barrier to viral infection in the adrenal gland, especially the cortex, were fewer in number than those found in the liver or spleen, suggesting that HSV-1 infection of the adrenal gland may result in suppression of local immunity. In this context, adrenal cell apoptosis would serve as a primitive type of immunity to limit viral replication (517).

After ip inoculation in mice, HSV-1 and HSV-2 replicated to high titers in the adrenal glands and the ovaries, but not in the testes (514, 530). In the adrenals, the lesions were mainly restricted to the zona fasciculata and the zona reticularis, sometimes extending to the medulla. In the ovaries, lesions were detected in follicles and in the stroma. During the course of infection, HSV nucleic acids and proteins could be detected in adrenal and ovary lesions, but there was no evidence of HSV latency in either organ (514, 530, 532).

After vaginal infections of mice with neuroinvasive strains of HSV-1 and HSV-2, virus replicates in the vaginal epithelium, in the paravaginal ganglia, in the spinal cord, and finally in the brain and the adrenal glands (516, 532). However, viral antigens could be demonstrated only in the medulla of the adrenal glands but not in the cortex (532). HSV

could not be isolated from liver, spleen, uterus, and ovaries. This is in contrast with the ip route of infection after which replication is occurring in different visceral organs including the adrenal cortex (516, 530).

After acute infection via intracameral inoculation, HSV-1 can establish latent infection in the adrenal gland, as demonstrated by the presence of latency-associated transcripts in adrenal tissues. Reactivation from latency is induced with appropriate stimuli (515).

The adrenal gland is a route of HSV-1 spread to the central nervous system after a viremic phase (528). After replication in the adrenal gland, the virus enters the preganglionic nerve fibers that supply the medulla and spreads through tracts of autonomic fibers in the thoracic cord (528). Efficient transfer into sympathoadrenal preganglionic neurons was also demonstrated after adrenal inoculation with nonreplicating mutant HSV-1 vectors, which, at variance with nonreplicative adenoviral vectors, remained confined to the adrenal gland (533).

C. Reovirus

Reovirus is selectively oncolytic for many tumor cell types, and this selectivity is inherent to the biology of the virus (534). The mechanism behind reovirus oncolytic activity was recently elucidated, with the demonstration of preferential reovirus replication in cells with an activated *ras* signaling pathway (535). When reovirus infects normal cells, early viral transcription activates double-stranded RNA-activated protein kinase, which inhibits protein translation by phosphorylation of EIF-2 α . This cellular response inhibits reovirus replication. In cells with an activated *ras* pathway, RNA-activated protein kinase phosphorylation and activity are impaired, thereby allowing viral protein synthesis and the lytic cycle to proceed.

The efficacy of reovirus as an oncolytic agent via either intratumor or systemic administration was demonstrated in several *in vitro* and *in vivo* models of tumor with an activated *ras* pathway, including glioblastoma (66, 536), Lewis and lung carcinoma metastases (537), breast cancer (538), ovarian and colon cancer (539).

Infections in humans are generally mild and restricted to the respiratory and gastrointestinal tracts. However, reovirus infection may be associated with significant cytotoxicity in endocrine cells and with the development of an autoimmune polyendocrine disease with autoantibodies to the pancreas, anterior pituitary, thymus, and gastric mucosa (540, 541).

1. Adrenal gland. Experimental infection of neonatal mice with reovirus 3 was characterized primarily by encephalitis, hepatitis, and pancreatitis. Although not as a major manifestation, mice also showed acute adrenalitis, characterized initially by foci of coagulative necrosis, which enlarged and became surrounded by leukocytic infiltration (542). Viral replication was demonstrated mainly in adrenocortical cells, but also in medullary and endothelial cells (542). Reovirus infection, associated with a marked cytopathic effect, was also demonstrated *in vitro* in the Y-1 mouse adrenal cell line (543).

2. Pituitary gland. Specific receptors for reovirus have been demonstrated in pituitary cell lines (544), thereby allowing efficient reovirus growth in pituitary cell cultures (545). Cytopathic effects are especially associated with reovirus type 3 infection (545). The presence of reovirus particles was also demonstrated *in vivo* in the anterior, but not posterior pituitary of mice infected with reovirus type 1 (546). In particular, virus particles were found in GH-producing cells from infected mice, leading to decreased GH blood concentration (546). Moreover, infected animals showed transient diabetes mellitus and developed autoantibodies directed against insulin, GH, and gastric mucosa of uninfected mice. Reovirus type 3, in contrast to reovirus type 1, did not induce autoantibodies to GH. By use of recombinant viruses, the genomic region responsible for the induction of autoantibodies to GH was identified as the S1 gene segment from reovirus type 1, which codes for the sigma 1 polypeptide (*i.e.*, hemagglutinin). At variance, virus containing the S1 gene segment from reovirus type 3 failed to infect cells in the anterior pituitary and did not induce autoantibodies to GH (546).

3. Endocrine pancreas. Efficient reovirus infection of the endocrine pancreas has been demonstrated in β -cell cultures (547) and *in vivo* (546, 548) in association with autoimmune diabetes mellitus (540, 546, 549). In particular, reovirus produced insulinitis when inoculated into 1- to 2-wk-old mice. Viral particles were observed in insulin-containing β -cells, but not in glucagon-containing α -cells. The infection resulted in β -cell destruction, reduction of the pancreatic insulin content, and impaired response to the glucose tolerance test (548). Reovirus infection of β -cells led to substantial cytopathic effects also *in vitro*, although without significant impairment of insulin secretion (545). The mechanism at the basis of reovirus-induced diabetes mellitus has not been completely elucidated; it seems to be mediated by T1-helper lymphocytes, whose production of IFN- γ may play a role in islet inflammation leading to cell destruction (550–552).

4. Thyroid gland. Newborn mice infected with reovirus type 1 developed a mild thyroiditis characterized by focal destruction of acinar tissue, infiltration of inflammatory cells, and autoantibodies to TG and microsomal antigens (553). If the study by Srinivasappa *et al.* (553) failed to detect viral antigens in thyroid tissue samples, with the exception of an occasional cell, Onodera and Awaya (554) showed viral antigens in the cytoplasm of thyroid epithelial cells and the presence of serum anti-TG autoantibodies. Reovirus type 3, in contrast to reovirus type 1, did not induce autoantibodies against TG. By the use of recombinants between reovirus type 1 and type 3, the segment of the reovirus genome responsible for the induction of autoantibodies to TG was identified to be S1, as observed for GH-secreting pituitary cells.

In vitro studies demonstrated that reovirus was able to infect and replicate in rat and murine thyroid cell lines inducing expression of both major histocompatibility complex (MHC) class I (555) and MHC class II antigens (556) in a dose-dependent fashion. Thus, reovirus infection might initiate autoimmune endocrine disease by inducing endocrine

cells to express MHC class II antigens and augmenting MHC class I antigen expression in susceptible animals. Reovirus types 1 or 3 enhanced expression of MHC class I and, to a lesser extent, of MHC class II molecules also on primary cultures of human thyroid follicular cells (557). The addition of antisera to IFN- α or IFN- β inhibited the increased class I MHC expression on thyroid follicular cells by both types of reovirus (557). These data suggest that the mechanism of MHC class I enhancement was most probably mediated by the release of IFN- α and IFN- β from infected cells.

5. Comment. Selective tropism of several viruses for endocrine glands, in particular the adrenal cortex, is impressive. Although experimental studies have demonstrated cytopathic effects in endocrine cells and autoimmunity associated with adenovirus, herpesvirus, and reovirus infection, it is also conceivable that, in most clinical infections, endocrine involvement is asymptomatic and not diagnosed. A finalistic explanation for adrenal tropism of viruses could be the favorable immunosuppressed environment created by local high cortisol levels. This hypothesis is corroborated by the demonstration of steroid hormone synthesis by a viral enzyme, *i.e.*, an active 3 β -hydroxysteroid dehydrogenase/ Δ 5- Δ 4 isomerase (3 β -HSD) encoded by the SalF7L vaccinia virus open reading frame, which can convert pregnenolone to progesterone (558). This enzyme, which has 31% amino acid identity to human 3 β -HSD, represents a virulence factor possibly involved in viral escape from cellular immune surveillance (559, 560). These conceptual implications should prompt the clinician to evaluate endocrine gland involvement in the course of viral infection (either wild-type or engineered) for early diagnosis of impairment (*e.g.*, adrenal failure in the case of acute adrenal or herpes viral infection).

VI. Endocrine Response to Viral Vector Delivery

Infections are regarded by the neuroendocrine system as stressors. In the face of stress, the function of the neuroendocrine system is to protect the homeostasis of the body by modulating the immune response. The neuroendocrine system constantly monitors and regulates the activities of the immune system. Conversely, the immune system needs the neuroendocrine system to help in determining the context of a perceived threat and setting the best way to respond (353).

Administration of a viral vector for gene delivery may be accompanied by a response of the neuroendocrine system, such as in the course of viral infections. Besides viral particles, therapeutic genes, such as cytokines, may also contribute to the activation of the neuroendocrine response, as discussed in *Section IV*. The HPA axis plays a major role in stress response, and various viral agents and their products have been demonstrated to activate the HPA axis (354–357).

A. HSV-based vectors

Both nonreplicative and replication-competent HSV vectors are used for cancer gene therapy, mostly by direct intratumor delivery for brain cancer. Although effects of vector delivery on the HPA axis have not been accurately investigated so far, experimental studies with wild-type HSV have

demonstrated that the virus induces a marked activation of the HPA axis. After corneal inoculation in rats with crude HSV-1 preparations from infected cells, the virus invaded the nervous system and replicated in the brainstem without clinical signs of disease (519). During this early, presymptomatic stage of the infection, in which very low virus titers were detected in the brain, a marked elevation in serum ACTH and corticosterone levels was observed (519). These elevated hormone levels failed to respond to stressful stimulation or to dexamethasone suppression. HSV-1-induced HPA axis activation was not associated with any tissue damage or inflammatory infiltrates in the brain, and no virus was isolated from the hypothalamus (519). HPA axis activation was dependent on an intact ventral noradrenergic ascending pathway that connects the brainstem and the hypothalamic paraventricular nucleus (561). Moreover, after corneal or intrahypothalamic inoculation, HSV-1 induced the expression of the IL-1 β gene in the brainstem and hypothalamus (562). At variance with cerebral administration, systemic (ip) inoculation of HSV-1 did not influence HPA axis responses (519), indicating that effects on HPA axis were mediated centrally and not by systemic mechanisms.

HSV-1 can acutely activate the HPA axis before and independently of any viral replication, because intraventricular inoculation of UV-inactivated purified virions caused a marked, but transient, increase in serum corticosterone and ACTH (520). Moreover, HSV-1-induced HPA axis activation was demonstrated to depend on a permissive action of circulating glucocorticoids and on host-derived brain IL-1 (520).

B. Adenoviral vectors

Preclinical and clinical studies with adenoviral vectors have clearly demonstrated the efficiency of this gene transfer system. However, adenoviral vectors elicit strong humoral and cellular immune responses that reduce the persistence of transgene expression (563–565) and, more importantly, may be associated with significant toxicity in rodents (566, 567) and in primates (9, 568, 569). Untoward events associated with vector administration may be related to direct cytotoxicity in target tissues or may reflect an activation of immune response cascades (570).

Administration of first-generation adenoviral vectors causes a rapid induction of IL-6, as well as other indicators of the acute-phase response, in mice, primates, and humans that experience toxicity (568, 571, 572). Induction of TNF- α , observed in early phases after vector delivery, has also been associated with the activation of immune response to adenovirus (572–576). Investigation of the *in vitro* response of human peripheral blood mononuclear cells after exposure to wild-type adenovirus, replication-deficient recombinant adenoviral vectors, and empty capsids demonstrated a significant and sustained stimulation of IL-6, GM-CSF, and a panel of α - and β -chemokines, including IL-8, Mip1- α , Gro- α , and Rantes (577). Induction of TNF- α by intact virions was low, although stimulation by empty capsids gave a significant and sustained response (577). Similarly, exposure to empty capsids was more effective than exposure to intact virions in cytokine induction. Although this *in vitro* model did not include the cell populations responsible for *in vivo* toxicity,

e.g., vascular endothelium or hepatocytes, it demonstrated that binding of virions/capsids to the cell surface activates cellular transduction mechanisms that stimulate transcription of proinflammatory cytokine genes.

C. Newcastle disease virus

Systemic inoculation of Newcastle disease virus in mice caused hypersecretion of ACTH and corticosterone under basal condition (578, 579). This effect was not caused by the virus itself or by the stress of the infection, but was mediated at least in part by the release of IL-1 from immune cells (578, 580).

Comment. Viral infection represents a stressful event that activates the HPA axis. This effect is elicited not only by replicating wild-type viruses, but also by structural viral proteins, thus suggesting that even administration of non-replicating viral vectors could activate an acute stress response. Indeed, fever, chills, tachycardia, hypotension or hypertension, *etc.*, are often observed in association with systemic viral vector delivery.

VII. Risk of Germ Cell Transduction and Present Ethical Concerns

A. Risk of germ cell transduction

1. *Adenoviral vectors.* The possibility of inadvertent exposure of gonadal cells to gene therapy vectors has raised safety concerns about germ line infection. With regard to the risk of adenoviral infection, CAR expression has been demonstrated in mouse germ cells (581), thus suggesting the possibility that adenovirus could infect germ cells. However, some *in vivo* experiments indicate that this risk is low. After *in vivo* injection of an adenoviral vector carrying the germ cell-specific protamine promoter fused to the β -galactosidase reporter gene into the left ventricular cavity of mice, no expression of the reporter gene was detected in developing spermatids or in mature epididymal spermatozoa (581). Primary germ cells cultured *in vitro* were also refractory to adenoviral infection (581, 582). Moreover, no transgene expression was detected in preimplantation embryos produced by *in vitro* fertilization with sperm exposed to adenoviral vector (582). Direct injection of adenoviral vector into the testis and epididymis of mice resulted in transgene expression only within the testis interstitium and not within the seminiferous tubules (582). At variance, intraprostatic injection of a replication-competent adenoviral vector led to high-level and persistent accumulation of viral DNA in prostate, testis, and liver (583). *In vitro* infection of mouse prostate-, testis-, and liver-derived cell lines demonstrated that the virus was indeed capable of replication in these mouse cell types, although with reduced efficiency relative to human cells (583). Despite vector persistence in male gonads, no evidence of germ line transmission was observed in embryos resulting from adenovirus-injected males mated to females 1 and 4 wk after injection (583). Adenoviral transduction of Sertoli cells and Leydig cells, but not germ cells, was demonstrated also in rats in both *in vitro* and *in vivo* experiments (584). Efficient gene transfer into spermatozoa was instead

obtained in pigs, after exposure of spermatozoa to adenoviral vectors (585). Of the two-cell to eight-cell embryos obtained after *in vitro* fertilization with adenovirus-exposed sperm, 21.7% expressed the transgene, whereas about 7% of piglets obtained after artificial insemination were positive for transgene expression (585).

The risk of infection of female germ cells with recombinant first-generation adenoviral vector was tested in the mouse model by injecting infectious particles directly into the ovary and by exposing naked oocytes to adenoviral particles before *in vitro* fertilization (586). A very large amount of transgene expression was detected in the thecal portion of the ovary, but not in oocytes. No transgene expression was detected in preimplantation embryos, in fetuses resulting from mating of mice with adenovirus-injected ovaries, and in morulae obtained from eggs fertilized after vector exposure (586).

After intravascular or periadventitial adenovirus-mediated gene transfer into rabbits, marker gene expression was found in various tissues, including the vasculature of testis and epididymis, but not inside the testicular tubules or germ cells (587). No inadvertent germ line transmission was detected after systemic tail vein administration of adenoviral vector in male and female mice (588).

2. *AAV vectors.* The risk of germ line transmission of vector sequences was investigated in four species of male animals (mouse, rat, rabbit, and dog) after im injection of recombinant AAV vector (589). There was a dose-dependent increase in the likelihood that vector sequences could be detected in gonadal DNA in mice and rats, whereas dog DNA extracted from semen was negative for vector sequences. In rabbits, analysis of both semen and testicular DNA showed the presence of AAV vector sequences in testes, which diminished with time, but not in semen DNA. Recombinant AAV vector sequences were localized to the testis basement membrane and the interstitial space, whereas no intracellular signal was observed (587). Similar findings were obtained after hepatic artery administration of recombinant AAV in rats and dogs (589). Attempts to transduce isolated murine spermatogonia directly with the AAV vector were unsuccessful (589). Human subjects injected im with an AAV vector showed no evidence of vector sequences in semen (10, 590).

3. *Retroviral vectors.* Retroviral transduction of germ line cells has been demonstrated, although it seems to be a rare event. Intraperitoneal injection of ecotropic murine leukemia virus into newborn mice led to infection of thecal cells (ovary) and Leydig cells (testis), but not of germ line cells (591, 592). Both cell types actively synthesized viral RNA and expressed viral antigens. Production of viral particles from thecal cells was also demonstrated (592), and this was probably responsible for vertical transmission of retrovirus by female mice (591). Retroviral transduction was also achieved in adult mouse and rat spermatogonial stem cells *in vitro* and *in vivo* by using several infection systems (593, 594). No germ line transmission occurred in sheep when retrovirus supernatant or producing cells were injected into embryos *in utero* at both low (595) and high titer (596).

It is, however, conceivable that the risk of germ cell transduction is negligible after direct intratumor injection of ret-

roviral vector particles or retroviral vector-producing cells, a common procedure in cancer gene therapy (597). In fact, after sc injection or direct intracerebral injection of retroviral vector-producing cells, the presence of retroviral vector sequences generally remained limited to the injection site, although vector DNA may be occasionally detected in draining lymph nodes (598). These results are in agreement with data obtained from biological monitoring of patients enrolled in cancer gene therapy clinical trials, which demonstrated the occasional presence of vector sequences in circulating peripheral blood leukocytes (88, 411). Vector sequences have also been detected in autopsy specimens from brain tissue, liver, kidney, and lung specimens (599), but, importantly, they have not been detected in any gonadal specimens from patients evaluated to date.

Although testicular germ cells of HIV-seropositive men are frequently found infected by the virus (600, 601), germ line transduction by pseudotyped recombinant lentiviral vectors during gene therapy has not been demonstrated so far (602, 603).

4. *HSV vectors.* The presence of wild-type HSV has been demonstrated in human spermatozoa (604); however, transduction of the germ line even with replication-competent HSV-based vectors has been excluded after local delivery in animal models (605, 606).

5. *Nonviral vectors.* Although nonviral vectors are considered safe tools for gene delivery in humans, data on the risk of gene transfer to the germ line are controversial. Systemic iv injection of plasmid DNA/cationic liposome complexes led to transient transgene expression in several tissues, including ovary (607), a finding not replicated by other studies (608). Liposome-mediated gene transfer was also achieved in sperm through direct intratesticular injection in rats (609).

B. Ethical issues

Ethical issues in human gene transfer research have been deeply discussed in the scientific literature, to which the reader is referred (610–629). In the field of somatic cell gene therapy, which might simply be considered as an extension of traditional medical interventions aimed at manipulating expression of genes, the debate involves the relative risks and benefits of treatment, the adequacy of preclinical studies, the selection of patients, the protection of their rights, and their proxies to informed consent, free withdrawal, and privacy. At variance with somatic gene therapy, techniques aimed at germ line genetic intervention produce clinical changes that may be transmitted to the offspring of the treated subject. This aspect is the main consideration that has led to proscribing human germ line gene therapy. Other important concerns with germ line gene therapy are related to uncertainties on the long-term effects of gene transfer to patients and their offspring and evaluation of the cost-effectiveness of this approach. Another important argument that has arisen against germ line therapy is that it would encourage the practice of genetic enhancement and eugenics, thus fostering discrimination and stigmatization of subjects with certain genetic traits. For all of these reasons, there is a general consensus that gene therapy, both somatic and germ line,

should be evaluated as a clinical tool used on behalf of seriously ill patients, and not as a eugenic program or as a tool for personal social advantage (624).

VIII. Summary

The prevalent clinical application of gene therapy is cancer, accounting for 68.5% of the protocols. A decade after the first clinical trial of gene therapy, unequivocal proof of clinical efficacy is still lacking. Besides efficacy, safety remains a critical issue that needs to be further addressed before including gene therapy as a standard of care in the management of cancer patients.

Effects related to therapeutic genes or gene transfer procedures on the endocrine system have rarely been assessed in experimental models and in clinical trials. Viral and nonviral vectors may exert direct cell and tissue injury in endocrine glands, as well as impairing hormone production. Much experience has been achieved with adenoviral vectors, which show a natural tropism for adrenocortical cells (9). Adenoviral infection of the adrenal gland results in degeneration, hemorrhage, inflammation, and impairment of response to ACTH (348).

Side effects of gene therapy are also mediated by the cascade of cytokines both induced after vector administration and delivered as therapeutic genes. The HPA axis plays a major role in stress response, and various viral agents and products thereof have been demonstrated to activate the HPA axis (354–357). Knowledge of the interplay between vector delivery and endocrine response may be useful in the choice and design of vectors with improved safety profile.

Endocrinology also has contributed to the field of gene therapy by providing knowledge on the mechanisms underlying regulation of gene expression. This knowledge has been transferred to the development of regulated and targeted vector systems, such as tamoxifen-regulated, glucocorticoid-regulated, and other steroid hormone-regulated systems. Moreover, genes specifically expressed in endocrine glands such as *NIS*, *NAT*, and *ssr2*, which play a significant role in the diagnosis and treatment of endocrine tumors, have been successfully exploited as therapeutic genes for tumors arising from tissues other than endocrine glands (261–264, 277–281, 284–286).

Because gene therapy of cancer is moving toward the use of oncolytic viruses as a means to provide a generalized treatment to patients suffering for a systemic disease, replication-competent vectors will become more similar to their wild-type counterparts. Virologists and gene therapists should therefore more carefully consider the molecular complexity of such viruses/vectors (*e.g.*, adenovirus, HSV, poxvirus) and the strategies these viruses have developed in the course of their evolution to evade the immune response and to persist in the host. For example, some herpesviruses encode for chemokine and chemokine receptors, which play critical roles in viral infection and replication and viral escape from the immune system (630–633). Such virally encoded functions are exploitable to increase inflammatory response when pursuing a sort of tumor vaccination strategy. On the other hand, when viruses are to be used for tumor vaccina-

tion purposes, attention should be paid to delete relevant immunosuppressive genes, such as for the case of 3β -HSD in poxvirus. Whereas viruses and vectors derived therefrom can be safely used when delivered to the patient through their natural route of infection, some safety concerns may arise when such viruses/vectors are administered at high doses through other routes. In this case, impairment of the endocrine system may occur either directly through infection of natural target glands or indirectly through an abnormal immune-inflammatory response. A full assessment of the endocrine function, which has never been considered so far, is highly warranted.

Acknowledgments

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