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Toxicities in Gene Therapy

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ABSTRACT *: Although there are still many technical difficulties to be overcome, recent advances in the molecular and cellular biology of gene transfer have made it likely that gene therapy will soon start to play an increasing role in clinical practice. However, safety issues are raised from vector system. It is not clear whether it is safe to incorporate genes into nuclear DNA. Little is known about the antigenicity of gene product which the immune system is encountering. In this review, some safety-related topics are introduced and discussed.*

I. INTRODUCTION

Gene therapy is defined as "introduction into human body of genes or cells containing genes foreign to the body for the purpose of prevention, treatment, diagnosis, or curing of disease" According to this definition, gene therapy includes administration of genetically modified, corrected somatic cells such as peripheral blood lymphocytes, or hematopoietic stem cells and direct administration of corrected genes into the target tissues in patients. However, administration of genetic material intentionally designed to improve or enhance metabolic, structural, or functional processes is not included. And, definition of gene therapy does not include administration of a vector targeted to the germ cells, with the intention of genetically modifying future generations (Danks, 1994).

Gene therapy field is one of the fastest growing areas of clinical research. As an example of how quickly this field has moved, the very first patient to receive gene therapy was treated September 1990 (Anderson *et al.*, 1990), with autologous lymphocytes that had been transduced with a retroviral vector encoding the human adenosine deaminase gene. As of January 2000, there are more than 300 gene therapy protocols either currently active or in review for a variety of different indications with over 3,000 patients treated worldwide (Blaese, 1997).

Although the initial clinical trials were focused mainly on correction of the monogenic diseases such as cystic fibrosis or ADA deficiency in leukocytes, gene therapy has expanded to include such applications as increasing tumor antigenicity through introduction of a foreign HLA haplotype, conferring resistance to chemotherapeutic agents through transfection of target cells with multidrug resistance genes, and the introduction of wild-type tumor suppressor genes in cancer (Table 1) (Crystal *et al.*, 1994; Bordignon *et al.*, 1995).

There are a variety of gene delivery systems under development (Table 2). Murine retroviral vectors, the most commonly used system in clinical trials, require a proliferating target cell to stably integrate and express the vector genes (Danos and Mulligan, 1988). The requirement for proliferation allows the potential for targeting tumor cells in organs where the resident tissues are generally non-proliferative. Use of retroviral vectors is limited by the inability to produce them in high titers. Adenoviral vectors are gaining popularity due to their high titer and efficient gene transfer into target cells regardless of the state of cellular pro-

Table 1. US human gene transfer protocols

Type of study	Number of protocols
Therapy-related	320
Single-gene disorders	43
Cancer	218
Infectious diseases (HIV)	30
Other diseases/disorders	29
Making	35
Non-therapeutic	2
Total	357

US National Institutes of Health, Office of Biotechnology Activities; Protocols approved as of December 1999.

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Table 2. Vectors under study as gene delivery vehicles

	Retroviruses	Adenoviruses	Adeno-Associated Viruses	Liposomes	"Naked" DNA
Potential Advantages	Integrate genes into host chromosomes, offering chance for longterm stability	Most do not cause serious disease; large capacity for foreign genes	Integrates genes into host chromosomes; cause unknown human diseases	Have no viral genes, so do not cause diseases	Same as for liposomes; expected to be useful for vaccination
Drawbacks of Existing Vectors	Genes integrate randomly, so might disrupt host genes; many infect only dividing cells	Genes may function transiently, owing to lack of integration or to attack by the immune system	Small capacity for foreign genes	Less efficient than viruses at transferring genes to cells	Inefficient at gene transfer; unstable in most tissues of the body

liferation, however, these vectors do not stably insert their genes into the genome of the target cell (Bischoff *et al.*, 1996). Non-viral gene transfer include unconjugated DNA (naked DNA), DNA conjugates (e.g. DNA complexed to antibody or ligands specific to cell surface receptors) and liposomes (DNA encapsulated in a lipid bilayer) (Davis *et al.*, 1993). These non-viral methods appear to be non-toxic, non-integrating vector delivery systems. However, safety issues are raised from adenoviral vector system so far. In this review, some safety-related topics are introduced and discussed.

II. SAFETY AND SHORT-TERM TOXICITY OF A NOVEL CATIONIC LIPID FORMULATION FOR HUMAN GENE THERAPY

A novel cationic lipid, dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium (DMRIE), has been substituted into the DNA-liposome complex with dioleoyl phosphatidylethanolamine (DOPE), which both improves transfection efficiencies and allows increased doses of DNA to be delivered *in vivo*. To determine whether the introduction of DNA-liposome complexes in vivo produced toxicity to major organ systems, several serum biochemical parameters were evaluated either after a single injection or after three separate injections made at 2-weeks intervals in mice and pigs by Nabel group of University of Michigan (San *et al.*, 1993). Analysis of serum enzymes and protein from liver, kidney, bone, and pancreas revealed no significant changes 10 days after a single injection (Table 3) or 14 days after three treatments administered at 2-week intervals (Table 4). Similar analyses were performed in pigs that received arterial gene transfer with DNA-liposome complexes, and no biochemical abnormalities were observed (Table 5). Pathology analysis of tissues from these animals showed occasional incidental changes unrelated to the introduction of DNA-liposome complexes (Table 6). The

Table 3. Evaluation of selected serum enzymes and chemistries before and after a single intravenous injection of HLA-B7 DNA-liposomes in mice

Plasmid (mg) DMRIE/DOPE (nmol)	0.5 1.5		5 15		50 150	
	Pre	Post	Pre	Post	Pre	Post
Albumin (g/dl)	3.4±0.4	3.3±0.3	3.3±0.4	3.2±0.3	3.1±0.1	3.3±0.2
Alk Phos (IU/liter)	146±21	152±13	164±14	170±17	172±8	193±23
Amylase (U/liter)	2,261±31	2,337±192	2,395±277	2,465±339	1,612±166	2,226±217
Bilirubin (mg/dl)	0.1±0	0.1±0	0.1±0	0.1±0	0.1±0.1	0.1±0
BUN (mg/dl)	20±4	25±2	35±6	26±3	24±9	20±0
Creatinine (mg/dl)	0.5±0.2	0.6±0.3	0.5±0.2	0.3±0	1.0±0.2	0.2±0
SGOT (IU/liter)	71±8	60±7	57±3	61±6	40±12	60±8
Total protein (g/dl)	6.3±0.8	5.2±0.2	5.4±0.8	5.4±0.6	5.0±0	4.5±0.2

Blood samples were obtained from BALB/c female mice (n=5) prior to intravenous injection (Pre) and 10 days after (Post) tail vein injection with the indicated concentrations of DNA-liposome complexes. From *Human Gene Therapy* 4: 785, 1993.

Table 4. Evaluation of selected serum enzymes and chemistries before and after three intravenous injection of HLA-B7 DNA-liposome complexes in mice

Plasmid (μg) DMRIE/DOPE (nmol)	0.5 1.5		5 15		50 150	
	Pre	Post	Pre	Post	Pre	Post
Albumin (g/dl)	3.4±0	2.9±0	3.1±0.2	2.90	3.2±0.1	3.2±0.2
Alk Phos (IU/liter)	152±8	113±10	161±16	124±14	170±8	182±11
Amylase (U/liter)	2,246±17	2,319±185	2,213±172	2,223±116	2,585±178	2,183±277
Bilirubin (mg/dl)	0.1±0	0.1±0	0.1±0	0.1±0	0.1±0	0.3±0
BUN (mg/dl)	21±3	18±3	37±7	26±3	26±10	22±3
Creatinine (mg/dl)	0.7±0	0.4±0.3	0.4±0.3	0.4±0.3	1±0	0.3±0
Phosphorous (mg/dl)	6.7±0.5	6.2±0.5	7.2±0.5	5±0.8	7.6±0.9	7.7±1.8
SGOT (IU/liter)	69±10	53±15	56±3	69±28	41±13	46±2
SGPT (IU/liter)	35±3	27±9	31±8	37±5	25±4	26±2
Total protein (g/dl)	6.3±1	4.8±0.3	5.4±0.9	5.1±0.5	5.0±0	4.8±0

Blood samples were obtained from BALB/c female mice (n=5) prior to intravenous injection (Pre) and 14 days after (Post) the third injection of DNA-liposome complexes at the indicated concentrations. From *Human Gene Therapy* 4: 785, 1993.

Table 5. Evaluation of selected serum enzymes and chemistries before and after arterial gene transfer of HLA-B7 DNA-liposome complexes in pig

Plasmid (μg) DMRIE/DOPE (nmol)	5 15		50 150	
	Pre	Post	Pre	Post
Albumin (g/dl)	3.2±0.4	3.2±0.2	3.3±0.3	3.4±0.3
Alk Phos (IU/liter)	298±62	186±29	241±97	206±51
Amylase (U/liter)	1,799±139	2,170±470	2,269±605	2,527±1,297
Bilirubin (mg/dl)	0.14±0.05	0.22±0.04	0.10±0	0.14±0.05
BUN (mg/dl)	8±3	9±3	6±1	10±2
Calcium (mg/dl)	9.3±0.2	9.3±0.4	9.5±0.6	9.5±1.0
Chloride (mEq/liter)	101±1	99±5	103±1	101±5
Creatinine (mg/dl)	0.9±0.1	1.1±0.1	0.8±0.1	1.2±0.2
Glucose (mg/dl)	111±57	134±54	102±56	116±22
LDH (IU/liter)	538±146	507±161	425±62	489±173
Phosphorous (mg/dl)	10.3±0.7	7.9±0.8	10.5±1.4	10.9±4.1
Potassium (mEq/liter)	5.1±1.0	4.4±0.8	4.5±0.4	4.8±1.1
SGOT (IU/liter)	28±8	443±0	27±13	43±30
SGPT (IU/liter)	29±14	42±12	36±9	40±7
Sodium (mEq/liter)	139±2	135±5	143±2	142±9
Total protein (g/dl)	5.0±0.1	5.4±0.5	5.7±0.5	5.7±0.5

Blood samples were obtained from pigs (n=10) prior to (Pre) and 17 days after (Post) arterial gene transfer of DNA-liposome complexes at the indicated concentrations (n=5 for each concentration). From *Human Gene Therapy* 4: 786, 1993.

incidental findings, including occasional peribronchial lymphoid aggregates in the lung or liver were seen in control animals that did not receive DNA-liposome treatments in previous studies. Similarly, no pathological abnormalities were detected after three treatments with DNA-liposome complexes in mice. These pathology studies demonstrated that the administration of DNA-liposome complexes intraarterially was well tolerated *in vivo*, with no adverse responses detected biochemically or in tissues.

And, they examined whether this formulation caused significant acute or chronic cardiac toxicity from this treatment. As an evaluation of cardiac toxicity, CPK measurements were performed in mice before or after injection with DNA-liposome complexes. No significant changes in CPK levels were noted pre- or post- injection (Table 7). In addition to these analysis, histopathological analysis of tissue was performed. No significant pathological abnormalities were detected at 14 days to 6 weeks after injection.

Table 6. Histological analysis of tissue following introduction of DNA-liposomes *in vivo*

Species: μg DNA/*nmol liposome*:	Mice			Pigs	
	0.5/1.5 (n=5)	5/15 (n=5)	50/150 (n=14)	5/15 (n=4)	50/150 (n=6)
Heart	Normal	Normal	Normal	Normal	Normal
Lung [a]Focal peribronchial lymphoid aggregates	2/5	Normal	3/14	3/4	3/6
Liver [a]Focal portal mononuclear inflammatory aggregates	2/5	2/5	4/14	Normal	3/6
Kidney [a]Focal interstitial mononuclear inflammatory cells	Normal	Normal	1/14	Normal	Normal
Spleen	Normal	Normal	Normal	Normal	Normal
Skeletal muscle	Normal	Normal	Normal	Normal	Normal
Nontransfected artery	ND	ND	ND	Normal	Normal
Ovary	Normal	Normal	Normal	Normal	Normal

ND, not determined.
[a]Minor pathologic changes of no clinical significance. From *Human Gene Therapy* 4: 786, 1993.

Table 7. Measurements of total CPK(U/L) before and after intravenous injection with DNA-liposomes in mice

Plasmid (μg): DMRIE/DOPE (nmol):	0.5 1.5	5 15	50 150
Pre-injection	339±122	156±44	156±44
Post-injection	189±63	163±44	163±44
	p≤0.79	p≤0.96	p≤0.31

Serum samples were obtained from BALB/c female mice prior to intravenous injection and 16 hr following injection of HLA-B7 liposome complexes (n=5, each dose). From *Human Gene Therapy* 4: 787, 1993.

III. CATHETER-MEDIATED PULMONARY VASCULAR GENE TRANSFER AND EXPRESSION

To evaluate the fe ility of gene transfer to the pulmonary vasculature, cationic liposomes and adenoviral vectors encoding a human placental alkaline phosphatase (hpAP) gene were delivered into a pulmonary artery of 24 pigs by percutaneous right heart catheterization by Nabel group of University of Michigan (Muller *et al.*, 1994). Pulmonary tissue was harvested within 20 minutes or 5, 14, or 28 days later and was analyzed for gene transfer and expression. Five days after exposure to liposomes or adenoviral vectors, transfer of DNA and expression of mRNA were demonstrated in transfected lung tissue. Recombinant alkaline phosphatase protein was observed in both the vasculature and in alveolar septa but not in the bronchi. Expression of hpAP protein was observed at 5 days, was diminished at 14 days, and was absent 28 days after gene transfer with both liposome and adenoviral vectors.

To determine whether delivery of cationic liposomes or adenoviral vectors into the pulmonary vasculature is associated with systemic toxicity, the histologic parameter was examined. No major adverse effects of gene expression were detected by histological examination of the transfected lung segments compared with control segments (Table 8). Gene transfer to the lung by either vector was not associated with significant histological changes 5, 14, or 28 days later in other organs, including carotid artery, heart, liver, spleen, kidney, skeletal muscle, ovary, and testes.

Biopsy specimens of contralateral lung, carotid artery, heart, liver, kidney, spleen, lower limb skeletal muscle, and ovary or testes were examined by light microscopy for pathological changes. Occasional minor abnormalities were observed in both experimental and control animals, including focal portal mononuclear inflammatory cells in the liver, focal tubular stasis and interstitial mononuclear inflammatory cells in the kidney, and splenic congestion. These findings were not clinically significant and were also observed in saline control pigs and normal control pigs.

In addition to the examination of organ pathology, they performed an analysis of serum enzymes that provides a biochemical indicator. Blood samples from pig transfected with cationic liposome or adenoviral vector were compared with samples from normal pigs that had no experimental manipulation and with samples from pigs injected with saline (Table 9). These samples revealed no statistical differences in liver function or in lipid, glucose, amylase, or serum electrolyte levels among the normal control, saline

Table 8. Analysis of organ histology after direct gene transfer to the pulmonary artery with liposomes and adenoviral vectors

Organ	Pathological Changes	Normal Control	Saline Control 5 Days	RSV-hpAP Liposomes			ADV-hpAP		
				5 Days	14 Days	28 Days	5 Days	14 Days	28 Days
Carotid atery	...	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Heart	Mild epicardial fibrosis*	Normal	Normal	Normal	Normal	Normal	Normal	Normal	1/2
Liver	Focal portal mononuclear inflammatory aggregates*	1/2	1/2	2/3	1/4	1/2	2/5	2/3	Normal
Kidney	Focal interstitial mononuclear inflammatory cell*	Normal	Normal	Normal	Normal	Normal	Normal	Normal	...
	Focal chronic inflammation in medulla*	...	...	...	...	...	...	...	1/2
Spleen	Splenic congestion	Normal	1/2	1/3	1/4	Normal	2/5	1/3	Normal
Skeletal muscle	...	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Ovary/ testes	...	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal

RSV-hpAP indicates cationic liposomes; ADV-hpAP, adenoviral vector. The number of pigs with positive findings relative to the total number analyzed within each group at the designated time point after gene transfer is indicated. *Minor pathological changes of no clinical significance. From *Circ. Res.* 75: 1039, 1994.

Table 9. Evaluation of selected serum enzymes and chemistries in normal pigs, saline control pigs, and pigs before and after gene transfer with liposome and adenoviral vectors

	Normal Control	Saline Control		RSV-hpAP		ADV-hpAP	
		Pre	Post	Pre	Post	Pre	Post
Albumin, g/dl	3.1	3.1	2.9	3.2±0.2	3.4±0.2	3.0±0.3	3.3±0.3
Alk Phos, IU/l	309	275	234	27±51	197±68	213±81	204±82
Bilirubin, mg/dl	0	0.1	0.2	0±0	0.1±0.1	0.1±0	0.2±0.1
BUN, mg/dl	11	8	11	7±0.8	11±3.2	7±1.8	12±3.5
Calcium, total g/dl	9.8	10.0	9.5	10.0±0.5	9.8±0.4	9.5±0.8	9.6±0.8
Chloride, mEq/l	108	106	107	102±5	101±5	98±5	103±4
Cholesterol, mg/dl	95	86	88	88±12	85±12	87±18	85±23
Creatinine, mg/dl	1.0	1.0	1.2	0.8±0.1	1.2±0.3	0.9±0.2	1.2±0.2
GGT, IU/l	34	32	31	33±7.5	34±8	33±6	31±11
Globulin, total g/dl	2.0	1.9	2.0	1.9±0.4	2.3±0.4	1.9±0.4	2.2±0.5
Glucose, mg/dl	106	126	113	94±34	104±38	65±27	81±39
LDH, IU/l	592	371	390	430±46	491±96	435±62	554±139
Phosphorus, mg/dl	8.8	8.8	8.6	9.7±0.8	9.2±3.6	8.6±1.5	7.9±1.0
Potassium, mEq/l	4.6	4.0	4.2	4.0±0.2	4.5±0.9	4.2±0.4	4.3±0.5
SGOT, IU/l	46	26	35	25±12	32±12	26±5	40±21
SGPT, IU/l	36	33	30	37±11	44±8	38±9	32±11
Sodium, mEq/l	145	143	139	141±6.4	140±7	135±8	140±3
Total protein, g/dl	5.2	5	5.4	5.0±0.3	5.7±0.5	4.8±0.4	5.5±0.6
Triglycerides, mg/gl	64	22	26	15±0.6	22±12	17±11	21±6

RSV-hpAP indicates cationic liposomes; ADV-hpAP, adenoviral vector; Pre, before gene transfer; Post, after gene transfer; Alk Phos, alkaline phosphatase; BUN, blood urea nitrogen; GGT, -glutamyltransferase; LDH, lactate dehydrogenase; SGOT, serum glutamic-oxaloacetic transferase; and SGPT, serum glutamic-pyruvic transaminase. Values are mean±SD. From *Circ. Res.* 75: 1039, 1994.

control, and the two experimental groups. There was a small rise in serum creatinine and urea in the saline control and experimental groups that not statistically significant. The small change in renal function was not associated with any detectable histopathologic change in the kidneys and may have been related to postcatheterization volume status. In summary, gene transfer to pulmonary arteries with liposome and adenoviral vectors was not associated with major organ histopathology or biochemical abnormalities

Table 10. Biochemical and hematological toxicity surveillance of the 10 cervical carcinoma patients treated (mean values±SD)

Test	Before injection	After injection
Hematological		
Hemoglobin (g/100 ml)	9.8±1.6	10.2±2.6
Total WBC ($\times 10^6$/1)	9.86±2.3	11.3±4
Platelet ($\times 10^9$/1)	400±101	457.2±211.5
Liver functions		
Total bilirubin (mol/1)	8.8±2.9	10.2±1.8
Alanine transferase (mol/1)	18.8±14	22.4±13
Alkaline phosphatase (mol/1)	114.6±78	156.8±107

From *Gene Therapy* 4: 783, 1997.

IV. PHASE I STUDY OF IMMUNOTHERAPY OF CUTANEOUS METASTASES OF HUMAN CARCINOMA USING ALLOGENEIC AND XENOGENEIC MHC DNA-LIPOSOME COMPLEXES

The objectives of this study were to examine the toxicities of employing the human HLA-A2, HLA-B13 and the murine H-2K genes to generate tumor regression in patients with different cancer types via DC-Chol/DOPE cationic liposomes (Hui *et al.*, 1997). The study was composed of two phaseI/II trials involving a total of 19 late-stage cancer patients. The patients were given four weekly injections of a DNA-liposome mixture directly into a cutaneous nodule. These procedures resulted in no significant clinical side-effects (Table 10). Analysis of serum biochemical parameters revealed no pattern of systemic abnormalities among the patients treated. The results of liver function tests remained within the normal range before and after injection of MHC DNA-DC-Chol/DOPE liposome complexes. In hematological tests, there was a slight drop in the mean hemoglobin level, and a slight elevation of the mean total white cell count and mean platelet after the treatment. However, these changes were statistically insignificant.

V. TNFα GENE THERAPY WITH MYELOID PROGENITOR CELLS LACKS THE TOXICITIES OF SYSTEMIC TNFA THERAPY

Chapman group of Henry Ford Health System, Michgan have showed that hTNFα-secreting 32Dc13 cells were capable of inhibiting the development of leukemia by 32Dp210 myeloid leukemia cells (Gautam *et al.*, 1999). However, the higher dose of transduced cells may also produce TNFα-mediated toxic side effects. Therefore, they examined whether the high dose of myeloid progenitor cells transduced with hTNFα cDNA induces cachexia, tissue toxicity, myelosuppression, or interference with the engraftment of BMT. They show that as high dose as 25×10^6 32DTNFα cells does not alter gain in body weight, induce histopathologic changes in lung, liver, kidney, or intestine, suppress HPC in BM, or interfere with the engraftment of BMT and hematologic recovery. These findings provide support for using TNFα gene therapy with transduced HPC for eradication of residual leukemia following BMT without the toxic side effects of systemic TNFα therapy.

The tissue toxicity response to intraarterial TNFα infusion is characterized by acute inflammation of the pulmonary interstitium, with intravascular thrombosis, hemorrhagic necrosis, renal tubular necrosis, and inflammatory cell infiltrates in liver and small intestine. They examined whether injection of 32DTNFα cells (25×10^6) produces any of these histopathologic changes in liver, kidney, lung, or intestine. TNF-secret-

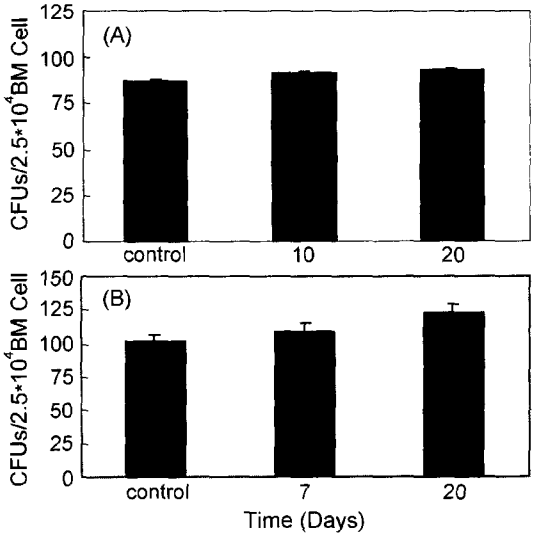

Fig. 1. Normal mice were injected with either 5×10^6 (A) or 25×10^6 (B) 32DTNF-α cells i.v. BM cells from uninjected control mice and mice injected with transduced cells were collected at different intervals as indicated. CFUs were determined by plating 2.5×10^4 BM cells in 1 ml methycellulose per Petridish (35 mm) in duplicate. Dishes were incubated at 37°C in 5% CO_2 for 7 Days, and CFU were scored under an inverted microscope. From *J. Hematother.* 8: 237, 1999.

Table 11. Effects of 32DTNFα cells on cellularity of spleen, BM, and peripheral blood

Mice	Spleen ($\times 10^6$)	BM ($\times 10^6$)	Peripheral Blood WBC ($\times 10^6$/ml)
Control	58±1.5	15.1±1.6	16.7±2.2
7 days	57±1.4	15.3±3.9	18.3±4.1
30 days	71±7.9	12.7±1.1	15.7±0.8

25×10^6 32DTNFα cells i.v. From *J. Hematother.* 8: 237, 1999.

ing cells produced no discernible histopathologic changes in any of these organs when examined 21 days after cell injection.

Whether TNFα secreted by transduced progenitor cells induces hematotoxicity was measured by determining CFU in the BM of mice injected with 32DTNFα cells. The results demonstrate that injecting mice with 5×10^6 (Fig. 1A) or 25×10^6 (Fig. 1B) transduced cells had no effect on the clonal growth of HPC or the number of CFU-total in the BM when examined 1 or 3 weeks after injection of transduced cells. Also, TNFα-producing cells (25×10^6) showed no effect on the cellularity of BM, spleen, or peripheral blood WBC counts (Table 11). Taken together, these findings indicate that myeloid progenitor cells transduced with hTNFα gene do not produce hematopoietic toxicity.

VI. ADENOVIRUS-MEDIATED P53 GENE TRANSFER IN ADVANCED NONSMALL CELL LUNG CANCER

Preclinical studies in animal models have demonstrated tumor regression following intratumoral administration of an adenovirus vector containing wild-type p53 cDNA (Ad-p53) by Jack Roth team of Texas MD Anderson Cancer Center (Swisher *et al.*, 1999). In a phase I clinical trial, they administered Ad-p53 to 28 patients with nonsmall cell lung cancer whose cancers had progressed on conventional treatments. As results, polymerase chain reaction (PCR) analysis showed the presence of adenovirus vector DNA in 18 (86%) of 21 patients with evaluable posttreatment biopsy specimens; vector-specific p53 messenger RNA was detected by means of reverse transcription-PCR analysis in 12 (46%) of 26 patients. Apoptosis was demonstrated by increased TUNEL staining in posttreatment biopsy specimens from 11 patients.

Vector-related adverse events were minimal (Table 12). No grade 4 toxicity was seen and grade 3 vector-related toxicity was limited to one incident of nausea after Ad-p53 injection. CT-guided administration of vector resulted in six pneumothoraces that were treated with percutaneous placement of a pigtail catheter in two patients and observation in four patients. Injection site pain was noted during 13 courses (15.5%) and resolved with oral pain medication in all patients. Four incidents of transient hemoptysis were noted after bronchoscopic injection and were resolved with observation. The most common vector-associated adverse event was fever, occurring 6~24 hours after injection in 23 treatments (27.4%). These fevers were treated with antipyretics or observation and resolved within the next 24~48 hours. There was no increase in adverse events with repeat treatments or higher doses of Ad-p53, and dose-limiting Ad-p53 toxicity was not reached in this trial. In addition, no patient demonstrated hypotension or anaphylaxis despite repeated (up to six) doses of Ad-p53.

Table 12. Adverse events associated with Ad-p53 (adenoviral vector) gene therapy in patients with nonsmall cell lung cancer*

Adverse event	No. of courses[≠]	Grade 1[#]	Grade 2[#]	Grade 3[#]	Grade 4[#]	Total[§]
Fever	84	13 (15.5)	10 (11.9)	0	0	23 (27.4)
Injection site pain	84	6 (7.1)	6 (7.1)	1 (1.2)	0	13 (15.5)
Pneumothorax	84	3 (3.6)	2 (2.4)	1 (1.2)	0	6 (7.1)
Nausea	84	3 (3.6)	0	1 (1.2)	0	4 (4.8)
Hemoptysis	84	2 (2.4)	2 (2.4)	0	0	4 (4.8)
Chills	84	1 (1.2)	1 (1.2)	0	0	2 (2.4)
Anorexia	84	1 (1.2)	0	0	0	1 (1.2)

*Toxicity defined by National Cancer Institute Common Toxicity Criteria (grades 1-4).
[≠] Total number of courses of Ad-p53 administered during the trial.
[#] Highest grade toxicity associated with Ad-p53 treatment. Percentage of courses with this level of toxicity is shown in parentheses.
[§] Total number of each adverse event (grades 1-4) associated with Ad-p53. Percentage of courses with the adverse event is shown in parentheses. From *J. Nat. Cancer Inst.* 91: 763, 1999.

VII. CHRONIC BRAIN INFLAMMATION AND PERSISTENT HERPES SIMPLEX VIRUS 1 THYMIDINE KINASE EXPRESSION IN SURVIVORS OF SYNGENEIC GLIOMA TREATED BY ADENOVIRUS-MEDIATED GENE THERAPY

Lowenstein group of University of Manchester, UK assessed the long-term outcomes of adenovirus-mediated conditionally cytotoxic gene therapy in a syngeneic glioblastoma model (Ginsberg *et al.*, 1990; Maron *et al.*, 1997; Dewey *et al.*, 1999; Kielian and Hichey, 1999). They implanted CNS-1 tumor cells which accurately reflect the infiltrative tumor growth observed in human gliomas, into the striata of Lewis rats, and then injected adenovirus expressing HSV-1-TK, and systemic ganciclovir. The treatment was very efficient, resulting in the survival of 80~100% of rats for at least 3 months. Unexpectedly, the brains of long-term survivors showed the presence of chronically active brain inflammation, as well as very strong and wide-spread HSV-1-TK immunoreactivity.

They perfusion-fixed long-term (90 days) survivors in thier experimental syngeneic glioma trials, and analyzed their brains histopathologically for the distribution of glial, inflammatory, and immune cell markers, as well as for the integrity of myelin fibers and oligodendrocytes. Sections stained with hematoxylin and eosin showed the presence of inflammatory infiltrates, and lateral ventricle enlargement ipsilateral to tumor and viral vector injection.

Immunohistochemical staining for the astrocyte markers vimentin and glial fibrillary acidic protein (GFAP) indicated widespread activation of astrocytes. GFAP is expressed by astrocytes, and is upregulated after activation. Vimentin is undetectable in resting adult rodent astrocytes, but is also upregulated after activation. Astrocyte activation was bilateral, but was strongest in the ipsilateral subcortical white matter. Vimentin-positive cells had typical astrocytic morphology, with perivascular end-feet. The distribution of activated GFAP immunoreactive astrocytes was much wider than the area occupied by vimentin-immunopositive cells. Astrocyte activation was present in all rats.

Activated ED1 immunoreactive macrophages/microglia were present mainly ipsilaterally, over a more restricted area than that occupied by activated astrocytes. Within the ipsilateral subcortical white matter, the area occupied by $ED1^+$, $CD3^+$, leucosyalin-positive, or $CD8^+$ cells overlapped with the hypercellularity detected in the sections stained with hematoxylin and eosin, GFAP or vimentin. Only very few could be detected in the contralateral subcortical white matter. Activated microglial/macrophages were also found within perivascular cuffs, with leucosyalin-positive, $CD3^+$ and $CD8^+$ lymphocytes. Lymphocytes were also present within the ipsilateral subcortical white matter, as well as infiltrating striatal tissue.

The loss of Luxol fast blue staining strongly indicated a substantial reduction of myelinated fibers in the ipsilateral subcortical white matter, which spread into its ventral extension. Luxol fast blue staining was less intense in the injected striatum than in the contralateral side, indicating actual loss of myelinated fibers within the striatum as well. Semi-thin Epon-embedded sections, stained with osmium and toluidine blue to highlight myelinated fibers, confirmed the loss of myelinated fibers within the subcortical white matter.

This study raises important points regarding current adenoviral-based clinical trials for human gliomas. There should be concern about the potential of this technique to induce chronic active inflammation which may ultimately have negative consequences on the functional integrity of the brain parenchyma. It will be important to determine whether these findings extend from the animal model to human brain tumors. Further study to identify the antigenic stimulus of this chronic inflammation is warranted to ascertain whether it will interfere with the administration of additional vector or the induction of a secondary immune response upon tumor recurrence.

VIII. FINDINGS OF THE DEATH OF JESSER GELSINGER

Preliminary findings from a thorough autopsy and exhaustive laboratory studies to discover the factors that led to the death of gene therapy clinical trial volunteer Jesse Gelsinger on September 17, 1999 by physician scientists at the University of Pennsylvania's Institute for Human Gene Therapy suggest that the adenoviral vector used in the trial initiated an

unusual and deadly immune system response that led to multiple organ failure and death (Yang *et al.*, 1994; Engelhardt *et al.*, 1994; Ye *et al.*, 1996; Paper *et al.*, 1998; Zimmer *et al.*, 1999). The trial (which began in April 1997) proceeded over a two-year period without major interruptions until the death of Jesse Gelsinger, the 18th patient to participate in Penn's ornithine transcarbamylase (OTC) gene therapy trial. A review of extensive animal studies performed prior to the launch of the clinical trial and the experiences of the previous 17 participants in the trial to treat OTC deficiency revealed no information that would have predicted the events that led to Mr. Gelsinger's death. There was no evidence for human error in his clinical management.

Overall, Mr. Gelsinger's clinical autopsy and laboratory study results indicate that the most significant factor in his death was oxygen deprivation brought on by Adult Respiratory Distress Syndrome (ARDS), a severe lung dysfunction not seen previously in any study animals or clinical trial volunteers. Immediately following vector infusion there was a diffuse activation of his innate immune system associated with fever. This was followed by an injury to the liver and inappropriate coagulation of blood; both processes began to resolve within 48 hours. His underlying genetic defect made it difficult for him to handle the stress of the immune activation, resulting in an accumulation of ammonia in the blood, and then coma. Mr. Gelsinger appeared to be improving three days after the vector until the onset of ARDS, which could not be reversed in time leading to his death.

Given the researchers' understanding (based on their own preclinical animal studies) that liver toxicity may be dose-related, and also given that the protocol was designed to deliver the adenovirus directly into the liver, the trial's principal investigators carefully monitored all patients for liver inflammation and/or injury using liver-function tests and liver biopsies. While there was evidence of liver inflammation in some patients, all such episodes proved transitory in nature (in that the inflammation self-corrected, with the patients' liver-function tests returning to baseline within two weeks); and little, if any, episodes of liver inflammation were seen in the three patients who immediately preceded Mr. Gelsinger in the study.

The specific lot of vector administered to Mr. Gelsinger has been retested and contract laboratories used to help evaluate its safety have been audited. The dose of vector administered to the patient and the DNA sequence of the corrective gene inserted into the vector were confirmed to be correct. An exhaustive characterization of the remaining sequences in the vector revealed variants, duplication of an area representing less than 0.4% of its total length. This variation was not unique to the lot administered to Mr. Gelsinger. The researchers' investigation has failed to reveal a specific characteristic of the vector lot that could have caused the complications observed in Mr. Gelsinger.

One unexpected finding was a marked abnormality in Mr. Gelsinger's bone marrow which, at the time of autopsy, revealed a total absence of precursors responsible for production of red blood cells and an abnormality in the state of maturity of the precursors that produce white blood cells. The relevance of the finding to Mr. Gelsinger's death is unclear, although it may reflect a problem with his bone marrow that predated the gene therapy experiment.

Reactions to adenoviral vectors are well known and include liver toxicity, the development of antibodies, difficulty breathing, inflammation, coagulation disorder, and fever. After receiving the E1 and E4-deleted adenoviral vector containing OTC gene, Jesse succumbed to problems of a different magnitude, a cascade of irrevocable events. He became jaundiced, showed high levels of two cytokines and evidence of a blood-clotting disorder, and died of acute respiratory distress and multiple organ failure.

Unexpectedly, researchers found afterwards that significant amounts of vector had traveled beyond the liver into the lymph nodes, spleen, and bone marrow, and also that Jesse's bone marrow was precursor-cell-depleted. This led some to speculate that an underlying condition, perhaps interaction of the adenovirus with parvovirus (of which there was evidence), might have contributed to the rapid, extreme and unstoppable immune reaction.

Other scientists questioned whether the vector was in fact replication-competent, while yet others believed that administering the vector into the hepatic artery created an exaggerated immune response. Jesse was one of two patients of a total of 18 who received the highest dose of vector, which most likely exceeded the

safe level for patients with this and perhaps other diseases.

IX. CONCLUSION

Preclinical studies in support of novel gene therapy should be designed to answer questions specific to the class of vector transduced, the intended route of administration, and the clinical indication and to provide an estimation of the risk for the clinical trial. The studies to determine safety are selected based on the body of information available and the specific issues to be addressed and should employ the best available technology and methods. Safety data may also be obtained from well-designed efficacy studies that address specific questions related to safety as part of the proof of concept. Whenever possible, safety data for gene therapy may also be obtained from studies in animal models of the human disease to determine the contribution of the underlying disease pathology or physiologic changes to the toxicity of the therapeutic approach.

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