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RESEARCH ARTICLE

An inventory of shedding data from clinical gene therapy trials

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Abstract

Viruses are the most commonly used vectors for clinical gene therapy. The risk of dissemination of a viral vector into the environment via excreta from the treated patient, a phenomenon called shedding, is a major safety concern for the environment. Despite the significant number of clinical gene therapy trials that have been conducted worldwide, there is currently no overview of actual shedding data available. In this article, an inventory of shedding data obtained from a total of 100 publications on clinical gene therapy trials using retroviral, adenoviral, adeno-associated viral and pox viral vectors is presented. In addition, the experimental set-up for shedding analysis including the assays used and biological materials tested is summarized. The collected data based on the analysis of 1619 patients in total demonstrate that shedding of these vectors occurs in practice, mainly determined by the type of vector and the route of vector administration. Due to the use of non-quantitative assays, the lack of information on assay sensitivity in most publications, and the fact that assay sensitivity is expressed in various ways, general conclusions cannot be made as to the level of vector shedding. The evaluation of the potential impact and consequences of the observations is complicated by the high degree of variety in the experimental design of shedding analysis between trials. This inventory can be supportive to clinical gene therapy investigators for the establishment of an evidence-based risk assessment to be included in a clinical protocol application, as well as to national regulatory authorities for the ongoing development of regulatory guidelines regarding gene therapy. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords shedding; clinical trial; retrovirus; adenovirus; adeno-associated virus; poxvirus; literature inventory

Introduction

Until now, over 1000 clinical gene therapy trials have been conducted worldwide. The majority of these trials aim at cancer treatment and to a lesser extent at the restoration of a genetic defect. In about 69% of all clinical gene therapy studies a vector of viral origin is being used, predominantly derived from adenovirus and retrovirus and to a lesser extent from adeno-associated virus (AAV), poxvirus like vaccinia virus and canary pox virus (http://www.wiley.co.uk/genmed/clinical/, overview 2006). A major environmental safety concern for the use of a vector of viral origin for gene therapy is the potential leakage of the vector into the environment via excreta from the patient. This phenomenon is called shedding. Depending on national regulations concerning the use of genetically modified organisms, shedding is an important issue in the regulatory application procedure for a clinical gene therapy protocol. If gene therapy is considered as deliberate release of a genetically modified organism into the environment, the

clinical investigator will have to perform an environmental risk assessment regarding the likeliness of shedding and its possible consequences. Historical data on shedding of a comparable vector would be of great benefit for evidence-based risk assessment. However, an extensive inventory of shedding data from clinical studies is currently not available. We have performed a literature study in order to generate an overview of shedding data from gene therapy trials that have tested the most commonly used viral vectors, i.e., retroviral, adenoviral, AAV and pox viral vectors. This overview gives an insight in the occurrence of shedding of these vectors in clinical practice and can offer support for environmental risk assessment in future clinical gene therapy applications.

Methods

Definition of shedding

In this project, shedding is defined as the dissemination of a gene therapy vector in any form into the environment, which includes contamination of other persons, through excreta from the treated subject or patient. Urine, faeces, sweat, saliva, nasopharyngeal fluids (including nasal and pharyngeal swabs and bronchoalveolar lavages), skin and semen were considered excreta. In trials using a route of administration other than injection into the circulation, blood and related products like serum, plasma and peripheral blood mononuclear cells (PBMCs) were taken into consideration as well. In general, these biological materials are not considered true excreta since they do not shed into the environment spontaneously. Nevertheless, in trials using local administration, dissemination of the vector into the circulation may turn blood into an unexpected potential source for contamination of hospital personnel involved in clinical monitoring or persons in the close environment of the treated patient. Therefore, blood and related products are also considered as excreta in trials using local administration.

The occurrence of replication-competent virus in excreta after gene therapy with a retroviral or replication-deficient adenoviral vector was considered as a special form of shedding. Replication-competent virus can either be present in the gene therapy product or generated in the patient through recombination with wild-type virus. Shedding of this vector-derived product can be a risk to the environment as well. For this specific form of shedding, blood and related products were included in the analysis regardless of the route of administration.

Literature search

PubMed was searched for articles on clinical gene therapy trials published until 31 July 2006. A limit was set on 'Clinical trial' as publication type and the type of virus was used as a keyword. Wildcards were used to cover as many publications as possible (e.g. 'adenovir*' as the keyword for adenovirus). Publications on clinical gene therapy trials, including therapeutic vaccination studies, were selected and screened for reporting on shedding analysis. Adenoviral vectors were split into two groups, namely replication-deficient adenoviral vectors and conditionally replicating adenoviral (CRAd) vectors. Figure 1 show the results of the literature search presented per vector. Overall, 260 relevant publications were selected and analyzed. Shedding analysis was reported in 102 (39%) of the selected publications. For retroviral vectors, details on shedding analysis were included in 27 (37%) out of 73 publications [1–27]. These articles were subdivided into 16 publications on *in vivo* retroviral gene therapy using retroviral vector-producing cells or direct administration of the retroviral vector [3,7,8,13–21,23,25–27] and 11 publications on *ex vivo* retroviral gene therapy treating patients with cells that had been transduced *ex vivo* with the retroviral vector [1,2,4–6,9–12,22,24]. For replication-deficient adenoviral vectors, 106 publications were found, of which 52 (49%) described shedding analysis. One article used as a publication on a retroviral vector was also used as a publication on a replication-deficient adenoviral vector [20]. Two publications did not provide the outcome of the analysis, resulting in a final number of 50 publications used for further analysis of shedding data [20,28–76]. For CRAd vectors, 11 (44%) out of 25 publications contained information on shedding analysis [77–87]. Five of the other publications described the testing for the occurrence of the vector in blood and related products only, while the CRAd vector was administered directly into the circulation. For this route of administration, blood was not considered as a source for shedding (see above) and therefore these articles were not included in the list of selected publications. For AAV vectors, 9 publications were found, of which 7 (78%) reported on shedding analysis [88–94]. Shedding analysis was described in 5 (11%) out of 47 publications for pox viral vectors, which were all on vaccinia vectors [95–99]. Two of these publications described the clinical testing of a replication-competent vaccinia vector [95,96]. Studies on a replication-deficient vaccinia vector and a replication-restricted vaccinia vector were reported in one [98] and two [97,99] publications, respectively. In total, 100 publications have been used to make an inventory of shedding analyses and data.

In some publications, especially on trials using a retroviral vector, the generation of an antibody response against the vector was used as a parameter for shedding. We have not included this type of analysis and have only regarded a direct measurement of viral genomic sequences, proteins or infectious particles as shedding analysis.

Results

Shedding assays

For shedding analysis, various types of assays or a combination thereof were found to be used. In general,

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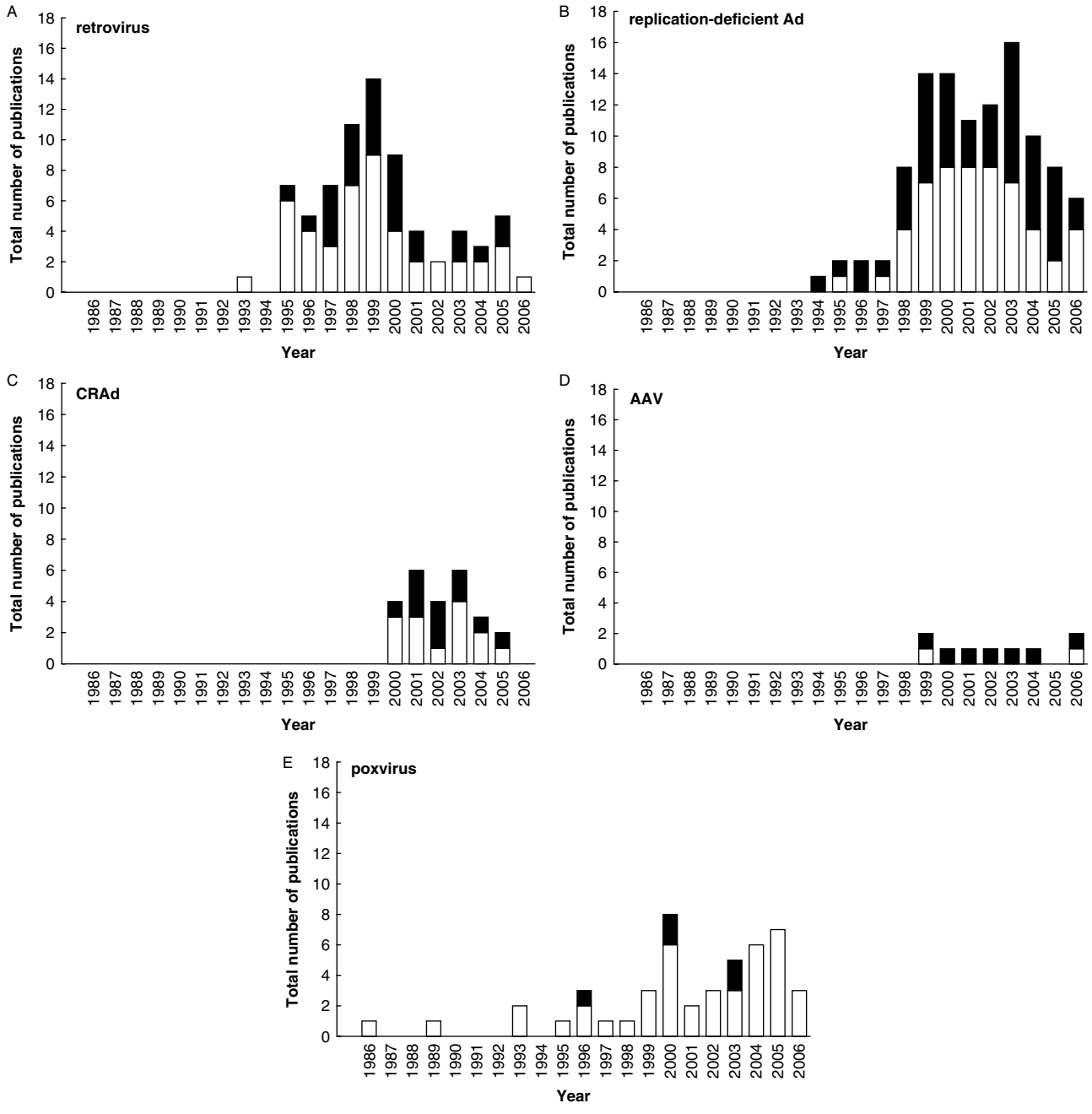

Figure 1. Publications on clinical gene therapy trials using a viral vector with information on shedding analysis. PubMed was screened for articles published until 31 July 2006 on clinical gene therapy trials using a retroviral vector (A), replication-deficient adenoviral vector (B), CRAd vector (C), AAV vector (D), or pox viral vector (E). The number of publications that have (solid bars) and have not (open bars) reported on shedding analysis is indicated. There is one article that has been used both as a publication on a retroviral vector and as a publication on a replication-deficient adenoviral vector [20]

analysis consisted of the assessment of vector genomic sequences by non-quantitative or quantitative polymerase chain reaction (PCR) and/or of infectious viral particles by a biological assay. The PCR specifically measured vector DNA through vector-specific primers in most cases.

The selected publications on *ex vivo* retroviral gene therapy all reported on the measurement of RCR only by either a PCR or a biological assay [1,2,4–6,9–12,22,24]. For *in vivo* retroviral gene therapy, shedding of the vector itself was in all cases assessed by PCR [3,7,8,13–21,23,25–27]. Biological assays such as the marker rescue assay and the PG4S^{+}L^{-} assay were used for the analysis of only replication-competent retrovirus (RCR).

For replication-deficient adenoviral vectors, an enzyme-linked immunosorbent assay (ELISA) for the detection of adenoviral proteins was used instead of PCR in some publications (Table 1) [29,32,49,54,58,59]. In about 40% of the cases, the shedding analysis was performed by a combination of two assays, primarily PCR and a biological assay. In 13 publications, this combination was used for the confirmation of a positive result, meaning that a

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positive signal in a biological assay was verified by PCR, and vice versa [28,30,32,34–36,47,53,59,67,68,73,74]. Infectious virus was mainly measured by a selective biological assay using a combination of 293 cells that enable replication of this type of vector and A549 cells that are not permissive to a replication-deficient adenoviral vector. In some publications, a flow cytometry based biological assay was described for this purpose. Replication-competent adenovirus (RCA) was assessed in a biological assay using cells that were insensitive to the replication-deficient adenoviral vector, such as A549 cells. In two publications, the assays used for shedding analysis were not specified, although test results were included [31,57].

In all publications on CRAd vectors, PCR was used for shedding analysis and in five cases this test was combined with a virus culture. Three out of these five publications reported that the two shedding assays were used as a combination for verification of a positive result [78,79,84].

Shedding of AAV vector was assessed either by PCR or by a biological assay. In the biological assay, samples were cultured in adenovirus-infected cells that express AAV rep protein and allow AAV vector replication, followed by PCR in the cell lysate for the detection of AAV genomes. In three publications, both PCR and a biological assay were used in an independent way [88,89,93]. The choice of assay depended on the sample to be tested. PCR was used for analysis of blood and related products and a biological assay for other excreta.

In all publications for pox viral vectors, shedding was assessed by virus culture using for instance Vero cells. In three publications describing both a PCR and a biological assay for shedding analysis, the two tests were used for verification of positive results [96,98,99].

Table 1. Types of assays used for shedding analysis

Assay	retrovirus (n = 27)	repl-def Ad (n = 52)	CRAd (n = 11)	AAV (n = 7)	poxvirus (n = 5)
Type of assay					
PCR	23 (85%)	31 (60%)	11 (100%)	5	3
• qPCR	3	6	5	0	0
• non-qPCR	14	13	5	5	0
• unknown	6	12	1	0	3
Biological assay	7 (26%)*	35 (67%)	5 (31%)	5	5
ELISA	0 (0%)	9 (17%)	0 (0%)	0	0
Unknown	2 (7%)	2 (4%)	0	0	0
Number of assays					
1 assay	18 (74%)	28 (54%)	6 (55%)	4	2
2 assays	5 (19%)	21 (40%)	5 (45%)	3	3
>2 assays	0 (0%)	1 (2%)	0 (0%)	0	0
unknown	2 (7%)	2 (4%)	0 (0%)	0	0

The number of publications using a certain type of assay is indicated. Percentages are based on the total number of publications per vector type. For PCR, a subdivision has been made into quantitative PCR ('qPCR'), non-quantitative PCR ('non-qPCR') or 'unknown' if the publication provides no details. Furthermore, the number of assay types used for shedding analysis is indicated for the publications analyzed.
For retroviral and replication-deficient adenoviral vectors, two publications did not provide details on the type of shedding assays used.
*Biological assays were only applied for the assessment of RCR.

Biological samples used for shedding analysis

Table 2 presents an overview of biological samples tested for vector shedding. In general for the four viral vectors, it can be stated that urine and blood and related products, in the case of local administration and analysis of replication-competent virus, are the most commonly tested biological samples. The choice of other biological samples appears to be related to the way of administration of the vector. For instance, skin samples are primarily analyzed in trials using intradermal injection, and nasopharyngeal fluids in trials using inhalation or intranasal administration. A notable observation is that for replication-deficient adenoviral vectors shedding is analyzed in a wide variety of biological samples, even when the vector is administered locally, which is the case in the majority of the publications. Blood and related products, urine, faeces and nasopharyngeal swabs are the most commonly tested samples for this type of vector. In contrast, for CRAd vectors blood and related products are the main samples

Table 2. Biological samples used for shedding analysis

Vector	Administration	Biological samples
Retrovirus *in vivo therapy* (16 publications)	ip (2), it (13), iv (1)	blood and related products (all publications) faeces (1), saliva (1), semen (1), skin (1), urine (2)
ex vivo therapy (11 publications)	id (2), it (1), iv (8)	blood and related products (all publications)
Replication-deficient adenovirus (50 publications)	ic (2), im (2), inh/in (9), ip (3), it (26), ivi (2), other* (6)	blood and related products (35), faeces (23), nasopharyngeal fluids (26), saliva (15), semen (2), skin (1), urine (44)
CRAd (11 publications)	ip (1), it (8), it + iv (1), it/ip (1)	blood and related products (9), skin (1), urine (3)
AAV (7 publications)	ia (1), im (1), inh/in (5)	blood and related products (5), faeces (4), nasopharyngeal fluids (3), saliva (4), semen (2), urine (5)
Pox virus (5 publications)	id (2), im (2), it (1)	blood and related products (3), faeces (1), nasopharyngeal fluids (2), saliva (1), skin (2), urine (3)

The number of publications is indicated in parentheses. Blood and related products include serum, plasma and peripheral blood mononuclear cells. Nasopharyngeal fluids include nasal and pharyngeal swabs and bronchoalveolar lavages.
Abbreviations: ic; intracoronary, id; intradermal, im; intramuscular, inh/in; inhalation/intranasal, ip: intraperitoneal, it; intratumoral, iv; intravenous, ivi; intravitreous
*Other routes of administration include intraarterial (1), intradermal (1), intramyocardial (1), intrapleural (2) and intravenous (1)

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used for shedding analysis. Furthermore, for retroviral vectors applied by *ex vivo* gene therapy the shedding analysis concentrated on the occurrence of replication-competent retrovirus (RCR) in blood and related products. The number of publications on AAV and pox viral vectors is limited, but there is a trend that shedding analysis is not restricted to blood and related products only. For AAV, five out of seven publications focused on gene therapy for cystic fibrosis through inhalation or intranasal administration [88,89,92–94]. In these trials, shedding analysis was performed in samples derived from the site of administration, such as saliva and nasal and pharyngeal swabs. Overall, only five of the 100 analyzed publications have reported on shedding analysis in semen [15,37,43,90,91].

Time schedule for shedding analysis

There is substantial variability in the timeframe of shedding analysis (data not shown) and therefore publications are difficult to compare in this respect. In general, for retroviral vectors analysis of vector sequences was performed in the first month after administration. In contrast, analysis of RCR was on average continued on a regular basis till 1 year after the treatment or beyond. In most publications both for replication-deficient adenoviral vectors and CRAd vectors, the shedding analysis was performed in the first month after administration. For AAV vectors, shedding was studied in the first weeks after administration and in one study was continued for 4 months [91]. For pox viral vectors, the shedding analysis appeared to be limited to the first 2 weeks only and the longest period of shedding analysis continued till 21 days after administration [95].

Shedding data

Overall, shedding data from 1619 treated patients have been collected (Table 3). It should be noted that the total number of included patients is higher, since in some publications a selection of patients has been tested for shedding. Although quantitative shedding data are described and information on assay sensitivity is provided in a number of the publications, the observed level of shedding is not discussed in this overview. Comparison of quantitative shedding data is hampered by the highly variable way quantity is expressed. For instance, the outcome of a PCR was defined as copy number, particles, particle-forming units (pfu) or positive cells, in most case related to a genomic DNA quantity, cell number or sample volume. As such, general conclusions cannot be made as to the level of vector shedding. Since it is not possible to discuss the shedding data for each individual publication in this overview, a summary of the observations is presented per vector type. The publications in which the assay sensitivity is described and/or quantitative shedding data are reported are listed as well.

Table 3. Patient numbers tested for shedding in the publications analyzed in this overview

Vector	Patients tested for shedding	
	Total number	Median per publication (min-max)
Retrovirus (27 publications)	445	12 (3–121)
in vivo therapy (16 publications)	*342*	*12 (6–121)*
ex vivo therapy (11 publications)	*103*	*7 (3–24)*
Replication-deficient adenovirus (50 publications)	869	14 (2–60)
CRAd (11 publications)	173	16 (3–30)
AAV (7 publications)	84	10 (3–23)
Poxvirus (5 publications)	48	9 (6–13)
Total all vector types	1619	12 (2–121)

The total number of patients (1619) that have been subjected to shedding analysis has been grouped per vector type. In addition, the median number of patients per publication is indicated with the minimum and maximum number in parentheses.

Retroviral vectors

All selected publications on *ex vivo* retroviral gene therapy trials focused on the occurrence of RCR in blood or related products only, as mentioned before. No RCR was detected in any of the 103 patients tested. Also for *in vivo* retroviral gene therapy, no RCR was observed in a total of 342 patients tested. In 10 out of 16 publications on *in vivo* retroviral gene therapy, the presence of vector in blood (primarily PBMCs) was demonstrated by PCR after intratumoral gene therapy for brain tumours [8,14,16,17,21,27], melanoma [3,7], and breast tumours [3], and intraperitoneal gene therapy for ovarian cancer [25,26]. Specifically, in 6 out of 8 publications describing gene therapy for brain tumours by intratumoral administration, vector sequences were found in PBMCs [8,14,16,17,21,27]. The duration of shedding varied from 1 to 28 days after administration. In none of these publications was the finding of vector genomic material confirmed as infectious viral particles. In one publication, semen from 13 patients was tested for the presence of vector at regular intervals up to week 53 after intravenous vector administration for the treatment of haemophilia A [15]. For one patient, a positive semen sample, being a 1 out of 10 replicate, was obtained at week 9. Since all previous and subsequent semen samples were negative, it could not be determined if this positive signal was present in the motile sperm fraction.

Overall, in 10 out of the 27 publications either quantitative shedding data were reported or information on the assay sensitivity was provided [2–4,7,8,12,15,17,19,20].

Replication-deficient adenoviral vectors

In 21 out of 50 publications (42%), shedding of vector was not observed after intratumoral [20,32,34,46,50,52,60, 61,67], intranasal or inhalation [31,39,45,71], intracoronary [37,42], intramyocardial [57], intravitreous [73],

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intraarterial [51], intrapleural [62,63] or intramuscular [75] administration. In addition, in one publication on gene therapy by intravitreous administration only RCA was measured with a negative outcome [72]. Five out of nine publications that did not find vector shedding in blood, urine or other excreta after intratumoral administration are related to the treatment of brain tumours [20,34,50,61,67]. A sixth publication on the same disease did show some shedding in plasma at one time point in 2 out of 7 patients [44].

The remaining 29 publications (58%) reported on shedding of vector DNA or infectious particles in various excreta, primarily depending on the route and site of administration and the time of analysis. For instance, saliva and nasopharyngeal fluids from some patients treated with a vector by intranasal administration or via inhalation were found to contain vector in a number of studies [28,47,55]. In a substantial number of publications (17 out of 26) describing intratumoral gene therapy in patients with various types of cancer shedding was demonstrated, primarily in blood and related products [30,33,35,36,43,44,48,49,53,54,58,59,64–66,68,74]. In general, shedding in blood was short lasting, peaking during the first hours and disappearing a few days after administration. In one of these studies, vector sequences were found in urine from the majority of treated patients up to day 32 after injection of the vector in prostate tumour cells [43]. The longest duration of shedding was reported in publications on the treatment of cystic fibrosis via inhalation [28] or of lung cancer using intrabronchial [38] or intratumoral administration [68]. After a single treatment, nasopharyngeal fluids like bronchoalveolar lavage, nasal and pharyngeal swabs and saliva were reported to be positive for vector sequences up to 21 [28], 30 [68] and even 90 days [38]. Two publications reported on the analysis of semen from 12 patients with angina pectoris 8 weeks after intracoronary administration [37] and from one patient with prostate cancer at day 14 after injection in a prostate tumour [43], respectively. The latter patient appeared to be positive for the vector based on PCR analysis.

Only a minority of the publications reported on the verification of a positive shedding finding. In 7 out of 8 publications, a positive PCR signal was confirmed as being derived from infectious adenoviral vector particles [30,35,36,47,53,68,74]. Eleven out of 50 publications on replication-deficient adenoviral vectors specifically studied the occurrence of RCA [20,30,31,38,39,50,53,57,64,65,72]. In none of the 201 patients tested could the presence of RCA be demonstrated.

In 18 out of the 50 publications either quantitative shedding data were reported or information on the assay sensitivity was provided [20,30,32,37,38,43–45,53,54, 64–67,70,71,75,76].

Taking the definition of shedding into consideration, the occurrence of the vector in the environment of the patient is an interesting parameter. Four publications were found describing shedding analysis in blood, faeces or throat swabs obtained from health care personnel who came into close contact with the patient [30,36,38,68]. Interestingly, in none of the individuals studied (up to 54 in one publication [68]) could the presence of the vector or RCA be demonstrated.

CRAd vectors

Three out of 11 publications describing an intratumoral application or an intraperitoneal administration reported a negative outcome for vector shedding in blood, urine or skin [81,84,87]. In the other 8 publications, the occurrence of vector DNA was demonstrated in blood by PCR after intratumoral administration of the CRAd vector [77–80,82,83,85,86]. The duration of shedding in blood ranged from a few hours to 76 days after administration. In two publications shedding results pointed to viral replication, demonstrated by a second peak of circulating viral genome [77,80]. In two publications it was studied if the presence of vector DNA in blood could be linked to shedding of infectious viral particles. No infectious viral particles were demonstrated [78,79]. In contrast, shedding of infectious vector particles was demonstrated in urine up to day 8 after vector injection in a prostate tumour [77].

In seven publications either quantitative shedding data were reported or information on the assay sensitivity was provided [77–80,83,85,86].

AAV vectors

Four of the five publications related to gene therapy for cystic fibrosis reported on shedding in nasopharyngeal samples and saliva during the first days after inhalation or intranasal administration [88,89,92,93]. Shedding in blood was rarely observed [88]. In the other two publications, the outcome of two clinical trials on gene therapy for haemophilia B was described. Intramuscular administration resulted in shedding of the AAV vector as measured by PCR in saliva and serum up to 24 and 48 h after injection, respectively, and no vector was observed in semen obtained after about 2 months [90]. When patients were treated with the same vector by infusion through the hepatic artery, dose-dependent shedding in urine was found during the first post-treatment week [91]. In 6 out of these 7 treated patients, vector DNA was found in semen up to 16 weeks after therapy. In one patient the vector was present in the seminal fluid and not in motile sperm [91].

In six publications either quantitative shedding data were reported or information on the assay sensitivity was provided [88–93].

Pox viral vectors

In all five publications studied, the replication-competent [95,96], replication-restricted [97,99], or replication-deficient [98] vaccinia vector was administered locally. No shedding was observed in blood, urine, faeces, saliva, nasopharyngeal samples and skin in four publications.

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One publication reported on the presence of live vaccinia virus in the detached scab from all eight treated patients after intradermal administration of a replication-competent vaccinia vector, but not in swabs taken from outside the injection site [96]. This was confirmed as the vaccinia vector by PCR. In this same publication, testing of wound dressings, hospital furniture and bed linen and air samples for the presence of vector was described [96]. Live vaccinia virus was only demonstrated in used dressings covering the injection site. In two publications information on the assay sensitivity was provided [96,99].

Discussion

This article presents an inventory of shedding data from publications on clinical gene therapy trials using retroviral, adenoviral, AAV and pox viral vectors. The literature search strategy was validated by a random test using the GEMCRIS database of American gene therapy trials. It appeared that the literature search strategy covered about 90% of the articles published on these clinical studies. Thus, we believe that a representative selection of relevant publications has been obtained and evaluated in this study.

Shedding analysis is reported in less than half (39%) of the publications found in our literature search. Data on shedding are not reported in the majority of publications on clinical gene therapy trials. In addition, the requirements for monitoring on the occurrence of shedding depend on national gene therapy regulations that are continuously adapted to previous experience and future development. Thus, the extent of availability of shedding data will vary between trials. In the articles that were found to report on shedding analysis, information on the set-up of shedding analysis as well as on the shedding data is often limited and the experimental design of shedding analysis varies significantly between trials. These observations suggest that shedding is not a prominent part of a report on the clinical outcome of gene therapy, while from the inventory it can be concluded that shedding of viral vectors is a common phenomenon for clinical gene therapy. Shedding mainly depends on the type of vector and the route of administration. For instance, in 36 out of 61 publications on adenoviral vectors, positive shedding results have been reported, in contrast to 1 out of 5 publications on pox viral vectors, while both vector types primarily were administered locally. Positive samples are often derived from excreta obtained close from the site of administration, such as nasopharyngeal samples and saliva after inhalation or intranasal administration [28,38,55,88,92], skin or scabs after intradermal administration [40,96], or urine after local administration in a prostate tumour [43,77]. It is of interest to note that local administration of retroviral and adenoviral vectors, primarily by intratumoral injection, in general is associated with shedding in blood. An exception is the finding that adenoviral vector shedding hardly occurs after injection in a brain tumour [20,34,44,50,61,67], suggesting that leakage of this vector type from this specific part of the body is an unlikely event. In contrast, shedding of retroviral vector to PBMCs after local administration in a brain tumour was reported in 6 out of 8 publications found. This is ascribed either to leakage of the vector or vector producing cells into the blood [27] or to the migration of locally transduced lymphocytes or tumour cells to the circulation [17,21,26]. Shedding is defined as the dissemination of a gene therapy vector into the environment via excreta from the treated patient. Since blood does not meet the criteria of excreta, potential dissemination of a vector through blood is often disregarded. However, the observations reported in this overview demonstrate that a viral vector can disseminate into the circulation after local administration. For hospital personnel involved in the clinical monitoring or treatment of a patient as well as persons in the close environment of the patient, blood can thus be a potential source of contamination if released deliberately during hospital procedures or by accident, as would also be the case for blood from a patient treated by direct administration of a vector into the circulation. This aspect has not been taken into consideration in this study. It is important to note that also in these cases blood can be a potential source for vector dissemination into the environment, although this is not regarded as shedding.

The effect of the administration route on vector shedding is most clear in two trials on AAV-mediated gene therapy for haemophilia B [90,91]. After intramuscular administration, shedding of the AAV vector was primarily observed during the first 2 days in saliva and serum and not in urine. The vector could not be detected in semen obtained after about 2 months [90]. In contrast, after infusion through the hepatic artery of the same vector, shedding was observed in urine during the first week. In addition, in 6 out of the 7 treated patients, vector DNA was found in semen up to 16 weeks after therapy [91]. Based on these observations, the FDA Biological Response Modifiers Advisory Committee (currently the Cellular, Tissue and Gene Therapies Advisory Committee) decided in 2002 that a positive semen test is no indication to put a clinical trial on hold, provided that the vector does not persist in semen longer than 1 year [100]. In that case, a trial will be halted in order to study the occurrence of germ-line transmission and to anticipate on the potential consequences of such an event. Furthermore, long-term monitoring of sperm was recommended by this committee.

It is difficult to draw conclusions on the duration of shedding in relation to the vector type. There are substantial differences between the publications with respect to factors affecting shedding analysis that complicate comparison of results, including the time schedule of shedding monitoring, type of shedding assay used, excreta tested, way of administration, dosage and dosage schedule. A number of observations on CRAd vectors indicate shedding after *in vivo* replication, which

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suggests that a replicating vector may shed for a longer period [77,80,86].

The generation of replication-competent virus in the patient is a major safety concern for replication-deficient vectors, especially for retroviral gene therapy. An important conclusion from this inventory is that until now replication-competent virus has not occurred in patients treated *in vivo* or *ex vivo* with a retroviral vector or with a replication-deficient adenoviral vector. This is based on results reported for 445 and 201 patients, respectively. Interestingly, for *ex vivo* retroviral gene therapy, treating a patient with cells that have been transduced with the vector *ex vivo*, shedding analysis only focuses on the assessment of RCR and not on the vector itself. According to investigators involved in this type of gene therapy, vector shedding is considered to be negligible by regulatory authorities due to the quality requirements regarding the absence of free vector particles in the treated cells.

Another safety issue of clinical gene therapy is spreading of a vector to the gonads, resulting in the risk of germ-line transmission. Shedding of vector in sperm can be an indication for biodistribution to the gonads. It appears that testing of semen is not a common practice in clinical gene therapy trials. In this inventory, five relevant publications are included of which three publications have reported on the occurrence of a retroviral vector [15], replication-deficient adenoviral vector [43] and an AAV vector [91] in semen. The presence of the retroviral vector in semen was explained by a false-positive result, since it concerned a 1 out of 10 replicate and previous and subsequent samples were negative [15]. The finding of adenoviral vector in the semen from one patient, which was the only sample collected [43], may be explained by the fact that the vector was injected intraprostatic and may have been excreted in semen via prostatic fluid. Notably, this sample was collected after 14 days while the sperm generation time in humans is 8 weeks [37]. Therefore, this shedding observation may not be related to biodistribution of the vector to the gonads. For the AAV vector, 6 out of 7 treated patients had positive semen samples. Based on the results of a study on one patient, it was demonstrated that the vector was present in the seminal fluid and not in the motile sperm fraction [91].

A crucial question is the relevance and implications of the positive shedding data presented in this overview. In principle, the moment of vector administration is the most critical time point in shedding since the environment is at that particular moment exposed to a high vector titer. The level of vector shed via excreta will be far lower. However, none of the publications have specifically addressed shedding analysis at this time point. It is important to note that in general positive shedding data are reported for only a part of the patients tested. In addition, shedding is often short lasting and is observed during the first 1 or 2 days after vector administration. Based on this inventory, it is hard to speculate on the level of shedding in general in the past clinical trials. In a number of the publications the assay sensitivity is defined or quantitative shedding data are reported. Nevertheless, since assay sensitivity is expressed in various ways, general conclusions as to the level of vector shedding could not be drawn. In addition, a positive PCR result points to the presence of vector DNA or RNA and is not necessarily indicative for potential harmful infectious viral particles. The occurrence of viral genomic material in excreta does in itself not pose a threat to the environment. Data on shedding of infectious particles would be of value in this respect, but availability of this type of information appears to be limited. None of the publications on *ex vivo* retroviral gene therapy reported on shedding analysis of infectious retroviral particles or vector genome, most likely due to the fact that the likeliness of vector shedding is considered negligible. In contrast, infectious retroviral particles can in principle be shed during *in vivo* retroviral gene therapy, although in the selected publications shedding analysis appears to be limited to vector genome testing by PCR only. In a limited number of publications on adenoviral vectors, PCR-positive samples were verified for the presence of viral particles using a biological assay. A number of these samples were confirmed to contain infectious particles in 7 out of 8 publications, indicating that shedding of intact adenoviral vector does occur in practice. In none of the publications on AAV vectors reporting on shedding of vector genomic material was this positive finding confirmed by a biological assay. The assessment of infectious AAV particles may be experimentally complicated by the fact that AAV is a non-cytopathic virus that depends on adenovirus for its replication. Nevertheless, in the majority of the publications excreta other than blood were tested for the presence of infectious particles only, indicating that the clinical use of a biological assay of AAV is feasible. Interestingly, for pox viral vectors the assessment of infectious particles appears to be common practice for shedding analysis and even is the first or only shedding assay carried out.

The potential impact of being exposed to an intact and infectious viral vector shed from an individual treated by gene therapy depends on the capacity of the vector to replicate and integrate as well as on the therapeutic transgene. For instance, the environmental threat imposed by shedding of a replication-deficient vector coding for a suicide gene, which has no effect in the absence of a prodrug, will be far less compared to shedding of a replication-competent vector coding for a cytotoxic gene. In addition, it is currently unknown if viral vector particles that are shed into the environment via excreta from treated patients are able to infect third parties and which critical level of shedding needs to be achieved in order to enable cross-contamination. In four publications that have reported positive shedding results, of which three publications described infectious particles in various excreta [30,36,96], tests of samples obtained from hospital personnel and furniture were all negative. However, it is most likely that the hospital safety measures taken in these studies were adequate to prevent cross-contamination. In one of these studies [96], describing

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intradermal administration of a vaccinia vector, live virus was observed in wound dressing while the vector was also found in the detached scab, indicating that the presence of vector in excreta may indeed result in contamination of the environment. There are historical data on adenovirus available indicating that inhalation of a minimum of 10E3 pfu of virus is sufficient to induce a clinically apparent infection of the respiratory tract [101]. Extrapolation of these data to contamination of humans with genetically modified adenoviruses via excreta is complicated and only preclinical studies may give an indication of the level of shedding of infectious particles required to induce clinical symptoms in third parties.

The inventory of shedding data presented in this overview is part of the outcome of a project entitled 'Inventory of shedding data and analyses: possibilities for standardization' that has been supported by the Commission on Genetic Modification (COGEM), a Dutch advisory body to the government on the risks to human health and the environment of the production and use of genetically modified organisms. The purpose of this project was to evaluate the possibilities for standardization of shedding analysis in clinical gene therapy trials in The Netherlands. Based on discussions with national and international academic and commercial gene therapy experts as well as experts from regulatory authorities, and the inventory of shedding data presented in this overview, it has been concluded that standardized shedding analysis is not realistic due to the current lack of uniformity in shedding analysis in clinical gene therapy studies. This is explained by differences between national environmental regulations concerning gene therapy as well as the lack of regulatory guidelines on shedding analysis. The COGEM has been recommended to develop a guidance or decision tree on shedding analysis that addresses the need to perform shedding analysis, the type of excreta that should be tested, the methods that could be used for analysis and the technical requirements for the shedding assays. Critical parameters for such guidance are the type of vector to be used, the therapeutic transgene and the way of administration. By offering such guidance to the gene therapy investigator, the degree of uniformity in shedding analysis between clinical trials may be increased, which will contribute to evidence-based environmental risk assessment.

Conclusions

Based on literature data on shedding of viral vectors during clinical gene therapy as presented in this overview, the following conclusions can be made:

- shedding of viral vectors occurs in practice;
- the majority of publications on clinical gene therapy trials do not report on shedding analysis;
- the occurrence of shedding mainly depends on the type of vector and the route of administration; and
- the relevance and implications of shedding remain a subject for further investigation.

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References

1. Abonour R, Williams DA, Einhorn L, *et al*. Efficient retrovirus-mediated transfer of the multidrug resistance 1 gene into autologous human long-term repopulating hematopoietic stem cells. *Nat Med* 2000; **6**: 652–658.
2. Amado RG, Mitsuyasu RT, Rosenblatt JD, *et al*. Anti-human immunodeficiency virus hematopoietic progenitor cell-delivered ribozyme in a phase I study: myeloid and lymphoid reconstitution in human immunodeficiency virus type-1-infected patients. *Hum Gene Ther* 2004; **15**: 251–262.
3. Braybrooke JP, Slade A, Deplanque G, *et al*. Phase I study of MetXia-P450 gene therapy and oral cyclophosphamide for patients with advanced breast cancer or melanoma. *Clin Cancer Res* 2005; **11**: 1512–1520.
4. Dunbar CE, Kohn DB, Schiffmann R, *et al*. Retroviral transfer of the glucocerebrosidase gene into CD34+ cells from patients with Gaucher disease: in vivo detection of transduced cells without myeloablation. *Hum Gene Ther* 1998; **9**: 2629–2640.
5. Hesdorffer C, Ayello J, Ward M, *et al*. Phase I trial of retroviral-mediated transfer of the human MDR1 gene as marrow chemoprotection in patients undergoing high-dose chemotherapy and autologous stem-cell transplantation. *J Clin Oncol* 1998; **16**: 165–172.
6. Kang WK, Park C, Yoon HL, *et al*. Interleukin 12 gene therapy of cancer by peritumoral injection of transduced autologous fibroblasts: outcome of a phase I study. *Hum Gene Ther* 2001; **12**: 671–684.
7. Klatzmann D, Cherin P, Bensimon G, *et al*. A phase I/II dose-escalation study of herpes simplex virus type 1 thymidine kinase "suicide" gene therapy for metastatic melanoma. Study Group on Gene Therapy of Metastatic Melanoma. *Hum Gene Ther* 1998; **9**: 2585–2594.
8. Klatzmann D, Valery CA, Bensimon G, *et al*. A phase I/II study of herpes simplex virus type 1 thymidine kinase "suicide" gene therapy for recurrent glioblastoma. Study Group on Gene Therapy for Glioblastoma. *Hum Gene Ther* 1998; **9**: 2595–2604.
9. Kohn DB, Bauer G, Rice CR, *et al*. A clinical trial of retroviral-mediated transfer of a rev-responsive element decoy gene into CD34(+) cells from the bone marrow of human immunodeficiency virus-1-infected children. *Blood* 1999; **94**: 368–371.
10. Malech HL, Maples PB, Whiting-Theobald N, *et al*. Prolonged production of NADPH oxidase-corrected granulocytes after gene therapy of chronic granulomatous disease. *Proc Natl Acad Sci U S A* 1997; **94**: 12133–12138.
11. Merrouche Y, Negrier S, Bain C, *et al*. Clinical application of retroviral gene transfer in oncology: results of a French study with tumor-infiltrating lymphocytes transduced with the gene of resistance to neomycin. *J Clin Oncol* 1995; **13**: 410–418.
12. Mitsuyasu RT, Anton PA, Deeks SG, *et al*. Prolonged survival and tissue trafficking following adoptive transfer of CD4zeta gene-modified autologous CD4(+) and CD8(+) T cells in human immunodeficiency virus-infected subjects. *Blood* 2000; **96**: 785–793.
13. Nemunaitis J, Fong T, Burrows F, *et al*. Phase I trial of interferon gamma retroviral vector administered intratumorally with multiple courses in patients with metastatic melanoma. *Hum Gene Ther* 1999; **10**: 1289–1298.

14. Packer RJ, Raffel C, Villablanca JG, *et al*. Treatment of progressive or recurrent pediatric malignant supratentorial brain tumors with herpes simplex virus thymidine kinase gene vector-producer cells followed by intravenous ganciclovir administration. *J Neurosurg* 2000; **92**: 249–254.
15. Powell JS, Ragni MV, White GC II, *et al*. Phase 1 trial of FVIII gene transfer for severe hemophilia A using a retroviral construct administered by peripheral intravenous infusion. *Blood* 2003; **102**: 2038–2045.
16. Prados MD, McDermott M, Chang SM, *et al*. Treatment of progressive or recurrent glioblastoma multiforme in adults with herpes simplex virus thymidine kinase gene vector-producer cells followed by intravenous ganciclovir administration: a phase I/II multi-institutional trial. *J Neurooncol* 2003; **65**: 269–278.
17. Rainov NG. A phase III clinical evaluation of herpes simplex virus type 1 thymidine kinase and ganciclovir gene therapy as an adjuvant to surgical resection and radiation in adults with previously untreated glioblastoma multiforme. *Hum Gene Ther* 2000; **11**: 2389–2401.
18. Ram Z, Culver KW, Oshiro EM, *et al*. Therapy of malignant brain tumors by intratumoral implantation of retroviral vector-producing cells. *Nat Med* 1997; **3**: 1354–1361.
19. Roth JA, Nguyen D, Lawrence DD, *et al*. Retrovirus-mediated wild-type p53 gene transfer to tumors of patients with lung cancer. *Nat Med* 1996; **2**: 985–991.
20. Sandmair AM, Loimas S, Puranen P, *et al*. Thymidine kinase gene therapy for human malignant glioma, using replication-deficient retroviruses or adenoviruses. *Hum Gene Ther* 2000; **11**: 2197–2205.
21. Shand N, Weber F, Mariani L, *et al*. A phase 1–2 clinical trial of gene therapy for recurrent glioblastoma multiforme by tumor transduction with the herpes simplex thymidine kinase gene followed by ganciclovir. GLI328 European-Canadian Study Group. *Hum Gene Ther* 1999; **10**: 2325–2335.
22. Simons JW, Jaffee EM, Weber CE, *et al*. Bioactivity of autologous irradiated renal cell carcinoma vaccines generated by ex vivo granulocyte-macrophage colony-stimulating factor gene transfer. *Cancer Res* 1997; **57**: 1537–1546.
23. Singh S, Cunningham C, Buchanan A, *et al*. Toxicity assessment of intratumoral injection of the herpes simplex type I thymidine kinase gene delivered by retrovirus in patients with refractory cancer. *Mol Ther* 2001; **4**: 157–160.
24. Sobol RE, Shawler DL, Carson C, *et al*. Interleukin 2 gene therapy of colorectal carcinoma with autologous irradiated tumor cells and genetically engineered fibroblasts: a Phase I study. *Clin Cancer Res* 1999; **5**: 2359–2365.
25. Tait DL, Obermiller PS, Redlin-Frazier S, *et al*. A phase I trial of retroviral BRCA1sv gene therapy in ovarian cancer. *Clin Cancer Res* 1997; **3**: 1959–1968.
26. Tait DL, Obermiller PS, Hatmaker AR, *et al*. Ovarian cancer BRCA1 gene therapy: Phase I and II trial differences in immune response and vector stability. *Clin Cancer Res* 1999; **5**: 1708–1714.
27. Colombo F, Barzon L, Franchin E, *et al*. Combined HSV-TK/IL-2 gene therapy in patients with recurrent glioblastoma multiforme: biological and clinical results. *Cancer Gene Ther* 2005; **12**: 835–848.
28. Bellon G, Michel-Calemard L, Thouvenot D, *et al*. Aerosol administration of a recombinant adenovirus expressing CFTR to cystic fibrosis patients: a phase I clinical trial. *Hum Gene Ther* 1997; **8**: 15–25.
29. Buller RE, Runnebaum IB, Karlan BY, *et al*. A phase I/II trial of rAd/p53 (SCH 58500) gene replacement in recurrent ovarian cancer. *Cancer Gene Ther* 2002; **9**: 553–566.
30. Clayman GL, el-Naggar AK, Lippman SM, *et al*. Adenovirus-mediated p53 gene transfer in patients with advanced recurrent head and neck squamous cell carcinoma. *J Clin Oncol* 1998; **16**: 2221–2232.
31. Crystal RG, McElvaney NG, Rosenfeld MA, *et al*. Administration of an adenovirus containing the human CFTR cDNA to the respiratory tract of individuals with cystic fibrosis. *Nat Genet* 1994; **8**: 42–51.
32. Dummer R, Bergh J, Karlsson Y, *et al*. Biological activity and safety of adenoviral vector-expressed wild-type p53 after intratumoral injection in melanoma and breast cancer patients with p53-overexpressing tumors. *Cancer Gene Ther* 2000; **7**: 1069–1076.
33. Dummer R, Hassel JC, Fellenberg F, *et al*. Adenovirus-mediated intralesional interferon-gamma gene transfer induces tumor regressions in cutaneous lymphomas. *Blood* 2004; **104**: 1631–1638.
34. Germano IM, Fable J, Gultekin SH, *et al*. Adenovirus/herpes simplex-thymidine kinase/ganciclovir complex: preliminary results of a phase I trial in patients with recurrent malignant gliomas. *J Neurooncol* 2003; **65**: 279–289.
35. Gilly FN, Sayag-Beaujard AC, Bienvenu J, *et al*. Gene therapy with AdV-IL2 (TG 1021) in unresectable digestive adenocarcinoma. Phase I-II study, first inclusions. *Adv Exp Med Biol* 1998; **451**: 527–530.
36. Gilly FN, Beaujard A, Bienvenu J, *et al*. Gene therapy with Adv-IL-2 in unresectable digestive cancer: phase I-II study, intermediate report. *Hepatogastroenterology* 1999; **46**(Suppl 1): 1268–1273.
37. Grines CL, Watkins MW, Helmer G, *et al*. Angiogenic Gene Therapy (AGENT) trial in patients with stable angina pectoris. *Circulation* 2002; **105**: 1291–1297.
38. Griscelli F, Opolon P, Saulnier P, *et al*. Recombinant adenovirus shedding after intratumoral gene transfer in lung cancer patients. *Gene Ther* 2003; **10**: 386–395.
39. Harvey BG, Leopold PL, Hackett NR, *et al*. Airway epithelial CFTR mRNA expression in cystic fibrosis patients after repetitive administration of a recombinant adenovirus. *J Clin Invest* 1999; **104**: 1245–1255.
40. Harvey BG, Worgall S, Ely S, *et al*. Cellular immune responses of healthy individuals to intradermal administration of an E1-E3- adenovirus gene transfer vector. *Hum Gene Ther* 1999; **10**: 2823–2837.
41. Hasenburg A, Tong XW, Rojas-Martinez A, *et al*. Thymidine kinase gene therapy with concomitant topotecan chemotherapy for recurrent ovarian cancer. *Cancer Gene Ther* 2000; **7**: 839–844.
42. Hedman M, Hartikainen J, Syvanne M, *et al*. Safety and feasibility of catheter-based local intracoronary vascular endothelial growth factor gene transfer in the prevention of postangioplasty and in-stent restenosis and in the treatment of chronic myocardial ischemia: phase II results of the Kuopio Angiogenesis Trial (KAT). *Circulation* 2003; **107**: 2677–2683.
43. Herman JR, Adler HL, Aguilar-Cordova E, *et al*. In situ gene therapy for adenocarcinoma of the prostate: a phase I clinical trial. *Hum Gene Ther* 1999; **10**: 1239–1249.
44. Immonen A, Vapalahti M, Tyynela K, *et al*. AdvHSV-tk gene therapy with intravenous ganciclovir improves survival in human malignant glioma: a randomised, controlled study. *Mol Ther* 2004; **10**: 967–972.
45. Joseph PM, O'Sullivan BP, Lapey A, *et al*. Aerosol and lobar administration of a recombinant adenovirus to individuals with cystic fibrosis. I. Methods, safety, and clinical implications. *Hum Gene Ther* 2001; **12**: 1369–1382.
46. Khorana AA, Rosenblatt JD, Sahasrabudhe DM, *et al*. A phase I trial of immunotherapy with intratumoral adenovirus-interferon-gamma (TG1041) in patients with malignant melanoma. *Cancer Gene Ther* 2003; **10**: 251–259.
47. Knowles MR, Hohneker KW, Zhou Z, *et al*. A controlled study of adenoviral-vector-mediated gene transfer in the nasal epithelium of patients with cystic fibrosis. *N Engl J Med* 1995; **333**: 823–831.
48. Kubo H, Gardner TA, Wada Y, *et al*. Phase I dose escalation clinical trial of adenovirus vector carrying osteocalcin promoter-driven herpes simplex virus thymidine kinase in localized and metastatic hormone-refractory prostate cancer. *Hum Gene Ther* 2003; **14**: 227–241.
49. Kuball J, Wen SF, Leissner J, *et al*. Successful adenovirus-mediated wild-type p53 gene transfer in patients with bladder cancer by intravesical vector instillation. *J Clin Oncol* 2002; **20**: 957–965.
50. Lang FF, Bruner JM, Fuller GN, *et al*. Phase I trial of adenovirus-mediated p53 gene therapy for recurrent glioma: biological and clinical results. *J Clin Oncol* 2003; **21**: 2508–2518.
51. Makinen K, Manninen H, Hedman M, *et al*. Increased vascularity detected by digital subtraction angiography after VEGF gene transfer to human lower limb artery: a randomized, placebo-controlled, double-blinded phase II study. *Mol Ther* 2002; **6**: 127–133.

52. Mundt AJ, Vijayakumar S, Nemunaitis J, *et al*. A Phase I trial of TNFerade biologic in patients with soft tissue sarcoma in the extremities. *Clin Cancer Res* 2004; **10**: 5747–5753.
53. Nemunaitis J, Swisher SG, Timmons T, *et al*. Adenovirus-mediated p53 gene transfer in sequence with cisplatin to tumors of patients with non-small-cell lung cancer. *J Clin Oncol* 2000; **18**: 609–622.
54. Palmer DH, Mautner V, Mirza D, *et al*. Virus-directed enzyme prodrug therapy: intratumoral administration of a replication-deficient adenovirus encoding nitroreductase to patients with resectable liver cancer. *J Clin Oncol* 2004; **22**: 1546–1552.
55. Perricone MA, Morris JE, Pavelka K, *et al*. Aerosol and lobar administration of a recombinant adenovirus to individuals with cystic fibrosis. II. Transfection efficiency in airway epithelium. *Hum Gene Ther* 2001; **12**: 1383–1394.
56. Rajagopalan S, Mohler ER III, Lederman RJ, *et al*. Regional angiogenesis with vascular endothelial growth factor in peripheral arterial disease: a phase II randomized, double-blind, controlled study of adenoviral delivery of vascular endothelial growth factor 121 in patients with disabling intermittent claudication. *Circulation* 2003; **108**: 1933–1938.
57. Rosengart TK, Lee LY, Patel SR, *et al*. Angiogenesis gene therapy: phase I assessment of direct intramyocardial administration of an adenovirus vector expressing VEGF121 cDNA to individuals with clinically significant severe coronary artery disease. *Circulation* 1999; **100**: 468–474.
58. Schuler M, Rochlitz C, Horowitz JA, *et al*. A phase I study of adenovirus-mediated wild-type p53 gene transfer in patients with advanced non-small cell lung cancer. *Hum Gene Ther* 1998; **9**: 2075–2082.
59. Schuler M, Herrmann R, De Greve JL, *et al*. Adenovirus-mediated wild-type p53 gene transfer in patients receiving chemotherapy for advanced non-small-cell lung cancer: results of a multicenter phase II study. *J Clin Oncol* 2001; **19**: 1750–1758.
60. Senzer N, Mani S, Rosemurgy A, *et al*. TNFerade biologic, an adenovector with a radiation-inducible promoter, carrying the human tumor necrosis factor alpha gene: a phase I study in patients with solid tumors. *J Clin Oncol* 2004; **22**: 592–601.
61. Smitt PS, Driesse M, Wolbers J, *et al*. Treatment of relapsed malignant glioma with an adenoviral vector containing the herpes simplex thymidine kinase gene followed by ganciclovir. *Mol Ther* 2003; **7**: 851–858.
62. Sterman DH, Treat J, Litzky LA, *et al*. Adenovirus-mediated herpes simplex virus thymidine kinase/ganciclovir gene therapy in patients with localized malignancy: results of a phase I clinical trial in malignant mesothelioma. *Hum Gene Ther* 1998; **9**: 1083–1092.
63. Sterman DH, Molnar-Kimber K, Iyengar T, *et al*. A pilot study of systemic corticosteroid administration in conjunction with intrapleural adenoviral vector administration in patients with malignant pleural mesothelioma. *Cancer Gene Ther* 2000; **7**: 1511–1518.
64. Stewart AK, Lassam NJ, Quirt IC, *et al*. Adenovector-mediated gene delivery of interleukin-2 in metastatic breast cancer and melanoma: results of a phase 1 clinical trial. *Gene Ther* 1999; **6**: 350–363.
65. Trudel S, Trachtenberg J, Toi A, *et al*. A phase I trial of adenovector-mediated delivery of interleukin-2 (AdIL-2) in high-risk localized prostate cancer. *Cancer Gene Ther* 2003; **10**: 755–763.
66. Tong AW, Nemunaitis J, Su D, *et al*. Intratumoral injection of INGN 241, a nonreplicating adenovector expressing the melanoma-differentiation associated gene-7 (mda-7/IL24): biologic outcome in advanced cancer patients. *Mol Ther* 2005; **11**: 160–172.
67. Trask TW, Trask RP, Aguilar-Cordova E, *et al*. Phase I study of adenoviral delivery of the HSV-tk gene and ganciclovir administration in patients with current malignant brain tumors. *Mol Ther* 2000; **1**: 195–203.
68. Tursz T, Cesne AL, Baldeyrou P, *et al*. Phase I study of a recombinant adenovirus-mediated gene transfer in lung cancer patients. *J Natl Cancer Inst* 1996; **88**: 1857–1863.
69. Wolf JK, Bodurka DC, Gano JB, *et al*. A phase I study of Adp53 (INGN 201; ADVEXIN) for patients with platinum- and paclitaxel-resistant epithelial ovarian cancer. *Gynecol Oncol* 2004; **94**: 442–448.
70. Zabner J, Ramsey BW, Meeker DP, *et al*. Repeat administration of an adenovirus vector encoding cystic fibrosis transmembrane conductance regulator to the nasal epithelium of patients with cystic fibrosis. *J Clin Invest* 1996; **97**: 1504–1511.
71. Zuckerman JB, Robinson CB, McCoy KS, *et al*. A phase I study of adenovirus-mediated transfer of the human cystic fibrosis transmembrane conductance regulator gene to a lung segment of individuals with cystic fibrosis. *Hum Gene Ther* 1999; **10**: 2973–2985.
72. Campochiaro PA, Nguyen QD, Shah SM, *et al*. Adenoviral vector-delivered pigment epithelium-derived factor for neovascular age-related macular degeneration: results of a phase I clinical trial. *Hum Gene Ther* 2006; **17**: 167–176.
73. Chevez-Barrios P, Chintagumpala M, Mieler W, *et al*. Response of retinoblastoma with vitreous tumor seeding to adenovirus-mediated delivery of thymidine kinase followed by ganciclovir. *J Clin Oncol* 2005; **23**: 7927–7935.
74. Fujiwara T, Tanaka N, Kanazawa S, *et al*. Multicenter phase I study of repeated intratumoral delivery of adenoviral p53 in patients with advanced non-small-cell lung cancer. *J Clin Oncol* 2006; **24**: 1689–1699.
75. Matyas L, Schulte KL, Dormandy JA, *et al*. Arteriogenic gene therapy in patients with unreconstructable critical limb ischemia: a randomized, placebo-controlled clinical trial of adenovirus 5-delivered fibroblast growth factor-4. *Hum Gene Ther* 2005; **16**: 1202–1211.
76. Tolcher AW, Hao D, de Bono J, *et al*. Phase I, pharmacokinetic, and pharmacodynamic study of intravenously administered Ad5CMV-p53, an adenoviral vector containing the wild-type p53 gene, in patients with advanced cancer. *J Clin Oncol* 2006; **24**: 2052–2058.
77. DeWeese TL, van der Poel H, Li S, *et al*. A phase I trial of CV706, a replication-competent, PSA selective oncolytic adenovirus, for the treatment of locally recurrent prostate cancer following radiation therapy. *Cancer Res* 2001; **61**: 7464–7472.
78. Freytag SO, Khil M, Stricker H, *et al*. Phase I study of replication-competent adenovirus-mediated double suicide gene therapy for the treatment of locally recurrent prostate cancer. *Cancer Res* 2002; **62**: 4968–4976.
79. Freytag SO, Stricker H, Pegg J, *et al*. Phase I study of replication-competent adenovirus-mediated double-suicide gene therapy in combination with conventional-dose three-dimensional conformal radiation therapy for the treatment of newly diagnosed, intermediate- to high-risk prostate cancer. *Cancer Res* 2003; **63**: 7497–7506.
80. Galanis E, Okuno SH, Nascimento AG, *et al*. Phase I-II trial of ONYX-015 in combination with MAP chemotherapy in patients with advanced sarcomas. *Gene Ther* 2005; **12**: 437–445.
81. Ganly I, Kirn D, Eckhardt G, *et al*. A phase I study of Onyx-015, an E1B attenuated adenovirus, administered intratumorally to patients with recurrent head and neck cancer. *Clin Cancer Res* 2000; **6**: 798–806.
82. Habib N, Salama H, Abd El Latif Abu Median A, *et al*. Clinical trial of E1B-deleted adenovirus (dl1520) gene therapy for hepatocellular carcinoma. *Cancer Gene Ther* 2002; **9**: 254–259.
83. Lu W, Zheng S, Li XF, *et al*. Intra-tumor injection of H101, a recombinant adenovirus, in combination with chemotherapy in patients with advanced cancers: a pilot phase II clinical trial. *World J Gastroenterol* 2004; **10**: 3634–3638.
84. Makower D, Rozenblit A, Kaufman H, *et al*. Phase II clinical trial of intralesional administration of the oncolytic adenovirus ONYX-015 in patients with hepatobiliary tumors with correlative p53 studies. *Clin Cancer Res* 2003; **9**: 693–702.
85. Mulvihill S, Warren R, Venook A, *et al*. Safety and feasibility of injection with an E1B-55 kDa gene-deleted, replication-selective adenovirus (ONYX-015) into primary carcinomas of the pancreas: a phase I trial. *Gene Ther* 2001; **8**: 308–315.
86. Nemunaitis J, Khuri F, Ganly I, *et al*. Phase II trial of intratumoral administration of ONYX-015, a replication-selective adenovirus, in patients with refractory head and neck cancer. *J Clin Oncol* 2001; **19**: 289–298.
87. Vasey PA, Shulman LN, Campos S, *et al*. Phase I trial of intraperitoneal injection of the E1B-55-kd-gene-deleted adenovirus ONYX-015 (dl1520) given on days 1 through 5 every 3 weeks in patients with recurrent/refractory epithelial ovarian cancer. *J Clin Oncol* 2002; **20**: 1562–1569.
88. Aitken ML, Moss RB, Waltz DA, *et al*. A phase I study of aerosolized administration of tgAAVCF to cystic fibrosis subjects with mild lung disease. *Hum Gene Ther* 2001; **12**: 1907–1916.

DOI: 10.1002/jgm

89. Flotte TR, Zeitlin PL, Reynolds TC, *et al*. Phase I trial of intranasal and endobronchial administration of a recombinant adeno-associated virus serotype 2 (rAAV2)-CFTR vector in adult cystic fibrosis patients: a two-part clinical study. *Hum Gene Ther* 2003; **14**: 1079–1088.
90. Kay MA, Manno CS, Ragni MV, *et al*. Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. *Nat Genet* 2000; **24**: 257–261.
91. Manno CS, Pierce GF, Arruda VR, *et al*. Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. *Nat Med* 2006; **12**: 342–347.
92. Moss RB, Rodman D, Spencer LT, *et al*. Repeated adeno-associated virus serotype 2 aerosol-mediated cystic fibrosis transmembrane regulator gene transfer to the lungs of patients with cystic fibrosis: a multicenter, double-blind, placebo-controlled trial. *Chest* 2004; **125**: 509–521.
93. Wagner JA, Messner AH, Moran ML, *et al*. Safety and biological efficacy of an adeno-associated virus vector-cystic fibrosis transmembrane regulator (AAV-CFTR) in the cystic fibrosis maxillary sinus. *Laryngoscope* 1999; **109**: 266–274.
94. Wagner JA, Nepomuceno IB, Messner AH, *et al*. A phase II, double-blind, randomized, placebo-controlled clinical trial of tgAAVCF using maxillary sinus delivery in patients with cystic fibrosis with antrostomies. *Hum Gene Ther* 2002; **13**: 1349–1359.
95. Baldwin PJ, van der Burg SH, Boswell CM, *et al*. Vaccinia-expressed human papillomavirus 16 and 18 e6 and e7 as a therapeutic vaccination for vulval and vaginal intraepithelial neoplasia. *Clin Cancer Res* 2003; **9**: 5205–5213.
96. Borysiewicz LK, Fiander A, Nimako M, *et al*. A recombinant vaccinia virus encoding human papillomavirus types 16 and 18, E6 and E7 proteins as immunotherapy for cervical cancer. *Lancet* 1996; **347**: 1523–1527.
97. Mukherjee S, Haenel T, Himbeck R, *et al*. Replication-restricted vaccinia as a cytokine gene therapy vector in cancer: persistent transgene expression despite antibody generation. *Cancer Gene Ther* 2000; **7**: 663–670.
98. Rochlitz C, Figlin R, Squiban P, *et al*. Phase I immunotherapy with a modified vaccinia virus (MVA) expressing human MUC1 as antigen-specific immunotherapy in patients with MUC1-positive advanced cancer. *J Gene Med* 2003; **5**: 690–699.
99. Scholl SM, Balloul JM, Le Goc G, *et al*. Recombinant vaccinia virus encoding human MUC1 and IL2 as immunotherapy in patients with breast cancer. *J Immunother* 2000; **23**: 570–580.
100. BRMAC. Minutes of the FDA Biological Response Modifiers Advisory Committee meeting 10 May 2002; Available: http://wwwfdagov/OHRMS/DOCKETS/ac/02/minutes/3855m2pdf.
101. Couch RB, Cate TR, Fleet WF, *et al*. Aerosol-induced adenoviral illness resembling the naturally occurring illness in military recruits. *Am Rev Respir Dis* 1966; **93**: 529–535.

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