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REVIEW

Gene transfer vector biodistribution: pivotal safety studies in clinical gene therapy development

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Techniques allowing for gene transfer vectors biodistribution investigation, in the frame of preclinical gene therapy development, are exposed. Emphasis is given on validation and test performance assessment. In the second part, specific gene vector distribution properties are reviewed (adenovirus, AAV, plasmid, retroviruses, herpes-derived vectors, germline transmission risks). The rationale for biodistribution by quantitative PCR, animal study and result interpretation is discussed. The importance and pivotal role of biodistribution study in gene transfer medicine development is shown through the determination of target organs for toxicity, germline transmission assessment and determination of risks of shedding and spreading of vectors in the gene transfer recipient and the environment.
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Introduction

In the field of gene transfer medicine, a range of vectors that have different properties might be used. On top of the intrinsic vector features, transgene and expression cassette characteristics as well as administration route and formulation can greatly influence toxicity potential. The aim of preclinical safety studies is to identify target organs for toxicity and reversibility if possible. In the field of gene transfer medicine development, and due to the wide variability of material that can be used for transduction of specific cell types, it is mandatory to perform, early in the development, dedicated studies that can answer to the question of spreading and target organs/tissues for toxicity. In small molecule's development, this is addressed by biodisponibility studies, which depend on the molecule tested. In gene transfer, quantitative PCR, which has become the standard, and is based on the genetic nature of the material transferred, can bring a lot of information. This information allows for further investigations into the target organs, germline transmission risks, shedding and spreading to the environment. This review will focus on technical and good laboratory practices for gene transfer vectors biodistribution studies, and on what is currently known of the distribution properties of the most used vectors to date.

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Biodistribution of the transferred genetic material: gene amplification techniques – technical considerations and requirements. Test characteristics and validation, current recommendations

Some and relatively few biodistribution studies have been published. A synopsis of a significant number of published studies is given in Table 1. These studies and some agencies' recommendations allow to help defining some technical guidelines for biodistribution. General toxicology rules apply that will help defining organs and fluids to be sampled. The general aim of biodistribution studies is defining the target organs for toxicity and the place where vector spread (intended or not), including germline transmission risk assessment. A quick look at Table 1 shows that in most studies the best irrigated organs are sampled (the liver, spleen, lungs, kidney, heart, brain). The injection site is also to be sampled (muscle, skin, etc) together with the draining lymph node(s) and their noninjected collaterals. In a lot of cases, some specific organs are studied, depending on the administration route, vector and transgene (see Table 1 and below).

Technique validation

Around one-fourth of the authors reviewed in Table 1 used real-time quantitative PCR (TaqMan, SybrGreen or Light Cycler), with or without transgene expression localization (which is more easy to perform in model studies with reporter genes). Other techniques include nonquantitative or semiquantitative PCR, FISH (approx. 50%), Southern blot[1,2] and reporter gene expression (LacZ,[1–6,7] luciferase,[8–11] and GFP[12]).

Overall, at least 75% of biodistribution studies published so far made use of genetic material detection

Table 1 Biodistribution study examples

Ref.	*Vector*	*Species*	*Technique*	*Route*	*Num admn*	*Dose*	*Vector titration*	*Duration*	*n prlvt*	*Num animals*	*Primer*	*Organs*	*Sensitivity*	*Notes*
8	AdV	Mouse	Semi-qPCR, Luciferase	i.v./ ivesic	2	10^9–5.10^9 PFU	PFU	72 h	5	3	tg	ki, ur, lu, adr, ov, bl, li, he	2500 copies	Model study
3	AdV	Rabbit	RT-PCR, LacZ, Histo	ivasc/ per	1	10^{10} PFU	PFU	D14	1	3	tg	ve, blo, mu, epi, te,li, he, lu, sp, lym, ki	ND	Arterial wall transfer
64	MLV	Rabbit	Taqman (dup), Histo	Eye inst	3	5×10^7 CFU	CFU bGa/Neo	D14	1	6	nd	br, lu, li, he, sp, ki, te, ov	10 copies	Corneal gt
35	Baculo/Adv	Rat	RT-PCR	i.cereb.	1	10^8 PFU	vg	D5	1	3	tg	sp, li, ki, lu, he, mu, br	ND	Baculo/AdV brain gt
41	AAV2	Mouse, rat, rabbit, dog	PCR (trip), FISH	i.m./ hepart	3	1.7×10^{11} 2.8×10^{13} vg/kg	vg	D90	2	3	tg	go, semen	1 copy/µg gen DNA	Germline transmission
60	HSV2	Mouse	Nested PCR (dup), ELISA	i.v.	1	10^6 PFU	TCID	D56	3	12	tg	blo, te, ov, li, br	0.02–2 PFU/µg DNA	Tox, safety en +, preclinical
18	HSV1	Aotus monkey	PCR (dup)	i.c.	1	3×10^7 PFU	PFU	D31–D730	>35	4	backb	sk, lym, lu, he, li, adre, sp, panc, go, ut, int, bm, br, sp cord	1–10 copies	Shedding, tox., preclinical
34	AdV	Mouse	PCR/Southern			Intraprostatic		10^{11} vp, 5×10^{11} vp/kg	OD	D29	2	20	tg	pro, te, bl, sev, li, emb
1	copy/75 ng DNA		Intraprostatic AdV				1							
57	pDNA	Mouse, rabbit	PCR	i.v., i.m.	1	50 µg	OD	D56	5	50	tg	blo, go, ln, li, sp, ki, he, lu, br, bm, mu(is)	100 copies	Nonviral distribution
4	AdV	Rabbit	RT-PCR, LacZ	i.m.	1	2×10^{10} PFU	PFU	D30	1	22	tg	mu, li, lu, sp, te	ND	VEGF transfer
9	AdV	Mouse	qPCR LC	i.p.	1	5×10^{10} vp	PFU, OD	D2	1	6	backb	li, sp, ki, he, lu, per, br, ov, blo	ND	Ovarian cancer
10	AdV	Mouse	qPCR LC	i.v.	1	5×10^{10} vp	vp	90 min	1	4	backb	lu, ki, li	ND	CAR ablation impact
39	AAV2	Monkey	PCR/Southern	i.m.	6	5×10^8 to 10^{10} i.p./kg	Dot-blot/RCA	18 months	2	8	tg	body fluids Shedding+AAV safety	100–1000 copies	
33	AdV	Rabbit	Taqman (trip) RT-PCR, Histo	ivasc	1	$1{,}15 \times 10^{10}$ PFU	PFU	D14	1	22	tg	aortic segment	ND	Restenosis-retargeted AdV
30	AdV	Mouse	PCR, Histo, GUSB expression	i.v.	1	10^8 PFU	PFU	D7	5	7	tg	li, sp, ki, he, br	ND	MPS VII AdV gt
32	AdV	Mouse, guinea-pig	Taqman (dup)	Umb. vein	1	3×10^8 PFU	PFU	24 h	1	21	tg	br, he, lu, li, ki, adr, sp, int, mu, pla	10.2 copies/µg gen DNA	AdV *in utero* gt
63	HIV-1	Mouse	Taqman (trip)	i.h.	1	5×10^7 TU	FACS	D8	8	20	backb	li, sp, thym, he, lu, in, go, ser, bm	1–10 copies/ 100 ng	LV *in utero* gt
31	AdV	Pig	Taqman (trip)	imyoc,	icoronary	1	10^{12} pu	ptc	24 h	1	1	tg	li,lu,he,sp	1/1,5E4 cells
	AdV myocardium													
40	AAV2	Baboon	Taqman	i.m.	2	5×10^{11}–5×10^{12} vg/kg	vg	4 months	1	4	tg	gonads	40 copies/1 E4 genomes	Alpha-antitrypsin
74	MVA	Mouse	Luciferase assay		Multiple	1	5×10^7 PFU	PFU	6–48 h	11	18	NA	ov, int, rect, lu, peyers, lym, sp	NA
	Vaccination													
17	AAV2	Rhesus monkey	PCR/ Southern/ Taqman (dup)	i.h.	1	8.6×10^{10} i.p.	Inf center assay	D30–D120	18	4	tg	he, lu, li, sp, ki, ton, int, sp cord, br, bla, int, adr, mu, nerve	10 copies/ reaction	*In utero* gt
42	AAV2	Dog	PCR	i.m.	1 (8–60 sites)	1.3×10^{11}–8.5×10^{12} pp/kg	Dot-blot	NA	7	5	tg	Swabs, serum, muscle	30 copies/ react.	Canine factor IX, shedding
11	LV (HIV/ ampho)	Mouse	PCR/ Luciferase	i.v.	3	10×10^6	rlu/ml	D7, D15, D21	9	9	tg	li, sp, he, lu, br, ki, mu, ov, bm	Luc: 10 fg	Targeted or untargeted LVs
15	AdV	Sigmodon hispidus	Taqman (dup)	s.c.	5+5 (D28–32)	6×10^8 (l.d.), 6×10^{10} (i.d.), 1.8×10^{12} (h.d.)	vp	D56	8	l.d.: 25, i.d.: 25, h.d.: 3	backb	br, he, lu, sp, li, ki, ov, sk	50 copies/ reaction	AdV in Cotton rats

23	pDNA	Mouse	PCR (trip)	i.d.	3	0.5–5 μg	OD	D30	10	61	backb	br, go, bm, sp, ki, li, lu, he, lymp nodes, skin	25 copies/ reaction	IL12 anticancer gt
56	pDNA	Mouse	Taqman/ Southern	i.v. aerosol	1	Aerosol: 2 mg. I.v.: 20 μg	OD	Aerosol: .5, 1, 2, 3, 24 h. I.v.: 1, 5, 10, 15, 30 min, 1, 2, 24 h	6	42	tg	lu, ki, ser, li, sp, he	ND	Aerosol, i.v. PEI pDNA
2	AdV	Mouse	Southern	i.v.	2	10^{11}	ptc (vp)	D3	4	6	NA	li, ki, sp, lu, he	ND	Anticancer conditionally replicating AdV
2	AdV	Mouse	Cy3-labeled vir	i.v.	1	10^{11}	ptc (vp)	15 min	1	ND	NA	li	ND	Anticancer conditionally replicating AdV
1	AdV	Rabbit	Southern, LacZ	i.v. aerosol	1	5×10^{11} i.p./kg	PFU	D5	7	2	NA	ov, sp, ki, lu, br, li, he	ND	AdV i.v. toxicology
5	Semliki	Mouse	RT-PCR, LacZ	i.v., i.m., s.c.	1	2×10^{7}	IU	D1	11	12	tg	sp, lym, ki, li, lu, br, he, col, ser, inj site	5 copies/ reaction	Route impact Semliki vector
58	pDNA	Mouse	RT-PCR	i.v.		hydrodynamic	1	1.3 mg/kg	OD	D7, D14, D30	6	30	tg	li, he, sp, ki, int, br
ND	Hydrodynamic gene transfer													
59	HSV-1	Aotus, marmosets	PCR	i.c.	1	$50–10^{4}$	PFU	6–90 (survival)	4	16	backb	ser, br, li, ki	200 copies/5 μl	Intracerebral HSV-1
7	AdV	Rat	Lac Z	Iliac vein	1	3×10^{11} vp	vp, iu, rca	D10	8	15	NA	li, sp, ki, he, lu, br, te, il	ND	AdV cirrhosis
13	pDNA, pox	Mouse	RT-PCR (dup)	i.d.	2	50 μg: pDNA; 10×10^{6}: MVA pox	OD, PFU	D46, D78	11	12	tg	sk, mu, te, ep, ov, lym, sp, br, he, ki, li	<100/μg gen DNA	Vaccination
62	LV. MLV	Mouse	Taqman (dup)	i.p.	1 (d2, 4,6, 8 after tum imp)	3×10^{6}	p24	Survival	4	24	tg	li, sp, lu, ki	ND	Ovarian cancer
44	AAV2	Rat	PCR	Local, i.m.	1	2×10^{10} (i.a.) 1.1×10^{11} (i.m.) (TU)	Inf center assay	D5, D11, D33	5	8 (i.a.) 4 (i.m.) 8 ctl	tg	joints, sp, thym, li, ser	ND	Experimental arthritis
6	LV	Mouse	Lac Z	i.v.	6	(delta = 12 h)	6×10^{7}–2×10^{8}	TU	84 h	8	9	NA	li, sp, br, he,lu, ki, duod, mus	ND
	Liver transduction													
20	pDNA	Mouse	qPCR	Local, i.v.	1	50 μg	OD	15 min, 24 h	6	10	Model	plasmid	he, lu, li, ki, sp, br	ND
	Vaccination													
19	pDNA	Mouse	qPCR (dup)	i.v.	1–6	50 μg	OD	15 min, 24 h	6	10	Model	plasmid	he, lu, li, ki, sp, br	ND
PEI	pDNA safety													
60	HSV-1	Monkey aotus, mouse	Taqman	Local	(prostate)	1	10^{6}–10^{7}	PFU				Monkey: 5 m; mouse: 21, 56 days	12	Monkey: 6, mouse:
	backb	br, sp cord, lym, adre, sp, te, pros, ur	100/μg gen DNA			Intraprostatic HSV								
12	LV (HIV/ VSVG)	Mouse	Taqman (dup)/ GFP	i.v.	1	2×10^{7}	TU	D4–D40	9	31	tg	go, bl, int, lu, he, ki, li, sp, br	1 copy/1 E5 cells	LV i.v. general safety study
55	pDNA	Mouse	PCR	i.m.	1	60 μg	OD	2 days, 1, 2, 3, 4 weeks	12	45	tg	plas, br, he, lu, li, ki, go, mu, lym	500/μg gen DNA	EGF antisens gt
16	Semliki/pDNA	Mouse, chicken	PCR	i.m.	1	pDNA: 25 μg; SFV: mouse: 1×10^{6}; chicks: 1×11^{7}	vp	SFV: mouse: 6, 16 h, 1, 3, 7, 10, 15 days; chicks: 2 h, 1, 3, 25 days. pDNA: mouse: 3, 7, 10, 14, 28, 93, 153, 246 days. Chicks: 2, 4, 6, 10, 17, 25 days	4	12	tg, backb	mu, li, sp, br, ki	1 DNA molecule	Vaccination

37	AAV2	Mouse	PCR	i.v.	1	10^{11}–5×10^{10}	vp	D154	5		tg	li, lu, sp, ki, he	ND	Factor IX
38	AAV2	Mouse	PCR/ expression	i.v.	1	1012	vp	D21, D56, D91	7	12	tg	li, ki, sp, he, lu, br, mus	ND	MPS VII AAV gt

Vector: AAV2, adeno-associated virus serotype 2; AdV, human adenovirus type 5; MVA, vaccine strain (poxvirus); LV, lentivirus; pDNA, plasmid DNA; HSV, hespes simplex virus; MLV, mouse leukemia virus.
Technique: qPCR, quantitative PCR; dup, in duplicate; trip, in triplicate; vir, virus.
Route: i.v., intravenous; ivesic, intravesical; ivasc, intravascular; per, periadventitial; inst, instillation; i.cereb., intracerebral; i.m., intramuscular; hepart, hepatic artery injection; i.c., i.p., intraperitoneal; umb, umbilical; i.h., intrahepatic; imyoc, intramyocardium; icoronary, intracoronary; s.c., subcutaneous; i.d., intradermal.
Dose and vector titration: PFU, plaque-forming units; CFU, colony-forming units; vg, viral genomes; TCID, tissue culture infecting dose 50; OD, optical density; vp, viral particles; RCA, recombinant-competent adenovirus; FCAS, fluorocytometer analyzer system; rlu, relative light units; IU, infectious units; ptc, particles.
Primer: tg, transgene; backb, backbone.
Organ abbreviations: ki, kidneys; ur: ureter; lu, lungs; adr, adrenals; ov, ovaries; bl, bladder; li, liver; he, heart; blo, whole blood; ser, serum; plas, plasma; mu, muscle; epi, epididymides; te, testes; sp, spleen; go, gonads; sk, skin; lym, lymphoid node; panc, pancreas; ut, uterus; int, intestine; bm, bone marrow; sp cord, spinal cord; pla, placenta; rect, rectum; col, colon; thym, thymus; duod, duodenum; pros, prostate; ve, vessel; ton, tongue.

and quantification techniques (Table 1), the technique of choice being quantitative PCR. Several considerations are to be taken into account when performing this kind of study for biosafety study purposes. As the aim is to determine the vector spread *in vivo*, there are PCR and *in vivo* study parameters that should be considered. Regarding the former, the most important parameters are sensitivity and specificity of the test, the use of spike or internal control to detect PCR inhibition, the replication of reactions (duplicates, triplicates) and the validation of the technique. Among the papers reviewed in Table 1, only three show evidence of a validated technique.[13,14,15] Table 2 shows the nature and amount of spiked material for some published studies, including Semliki virus-derived vector.[16] In the vast majority of published studies (Table 1), there was no spiked material. Some authors also used an internal control, which is often a competitive piece of DNA used in the same reaction tube.[12,15,17–20] The advantage of this technique is a precise control of inhibition in the same tube, the inconvenience being the sensitivity decrease because of competition. We would recommend qPCR in triplicate, with spiking of one of the reaction tubes with a low DNA amount (as in Hanke *et al*[13]). The sensitivity of the reaction in the presence of organ genomic DNA is also paramount. The determination of this parameter was not systematic in published studies (43% in Table 1), and even when determined, it was not often in the presence of organ DNA. To express sensitivity, the units used varied a lot, and the levels reached (the mean level is around 10–200 copies/reaction, Table 1). The sensitivity should be expressed as the number of copies per micrograms of genomic DNA. A sensitive technique should be in the range of 10–50 copies detected/μg genomic DNA, while FDA recommends <100 copies/μg genomic DNA. Regarding the repetition, we advocate for triplicate (five references in Table 1) with spiking, while some authors used duplicates (eight references, Table 1). In our experience, a 100% success rate at achieving <100 copies/μg DNA can easily be reached, while we detect 50 and 10 copies in 50 and 30% of analyses, respectively. It is important to distinguish between the limit of detection (LOD), which is the lowest detectable quantity, without precise quantification (because it is out of the linearity zone) and the limit of quantification (LOQ). The LOD can be less than 10 copies/μg DNA, while the LOQ varies between 10 and 100, depending on the run.

Choice of primers

In studies depicted in Table 1, 70% made use of transgene-specific primers. These are not strictly mandatory for biodistribution purposes, but they have more advantages than backbone-specific primers. The only advantage of the latter is that they can be used as generic primers for same vector studies. To achieve 10–100 copies/μg of genomic DNA sensitivity, it is highly advisable to design primers that amplify a small piece of DNA (less than 100 bp) and strictly avoid fragment sizes higher than 500 bp. Primers should also be optimized to preclude dimers and autoannealing. Primers and probes concentration should also be thoroughly optimized to increase sensitivity. Owing to increased risks of contamination, and difficulties in optimization and validation, we would not recommend nested PCR.

Table 2 Different qPCR internal control nature and amounts

DNA	*Amount*	*Notes*	*Reference*
Plasmid	1–100 copies	Several species	Arruda *et al*[41]
Vector plasmid	100 copies/μg	For urine and feces	Favre *et al*[39]
Vector plasmid	10 copies/sample	One of the triplicates	Hanke *et al*[13]
Vector plasmid	100 copies/sample	—	Imboden *et al*[23]
Vector plasmid	50 copies/sample	Shedding (humans)	Kay *et al*[45]
Plasmid	1–10^9 copies	Mouse, chicken	Morris-Downe *et al*[16]
Vector DNA	4–40 copies	NHP	Song *et al*[40]
Vector DNA	2.5×10^6 vector DNA/sample	Permissive species *(Sigmodon hispidus)*	Wildner and Morris[15]

Other parameters that should be considered for validation are precision (comparison of two sets of four replicates, with a variability under one standard deviation), repeatability and reproducibility. For quantitative PCR validation and optimization, see Lovatt.[21]

Organ sampling

Another important point is organ sampling. It should be carried out using disposable or sterile DNA-free separate sets of material to minimize contamination risks.[15,22] The order of organ sampling is also important. The injection site should be harvested at the end, beginning with the organs that are less likely to contain vectors.[23] For instance, when sampling after a tail vein administration, the liver is likely to contain significant amounts of genetic material, and should therefore be sampled among the last organs. To really study an organ biodistribution, it is also important to thoroughly wash the organs in PBS to decrease crosscontamination by body fluids (blood, etc). Pan *et al*[12] also proposed a 20 min perfusion at killing to minimize crosscontamination by blood. Organs to be sampled include the brain, lungs, heart, liver, kidneys, testes, ovaries, epididymides, prostate and the injection site (Table 1).[22] The extent of sampling is also a matter of debate, due to the heterogeneity of some organs (lungs, spleen, for instance).[24] The FDA recommends three different sections per organ or a representative homogenate.

Animal study design

The animal study design includes choice of species, number of animals and gender, age of animals, vector doses, batches and controls, duration and administration route. In the data presented in Table 1, half of the studies were carried out in mice (27), four in rats, six in rabbits (90% in rodents and lagomorphs), five in monkeys and two in dogs. The number of animals tested in biodistribution studies varies widely (see Table 1: 3–61). It is advisable to test between three and five animals of each sex per condition. The age of animals is around 6–8 weeks generally for rodents, and only reasons related to the forthcoming clinical protocol should determine a different choice. Doses and titration techniques are given in Table 1 to show that on top of having standardization issues, techniques and units used for viral vector titrations vary quite widely. Of special importance is the quality control and standardization of viral titration (using, when possible a reference material: universal for AdV, or in-house at least for other vectors). For biodistribution, we would recommend to test a high dose (far higher than what will be tested in the clinic) to really be able to define and detect what would be the target organs for toxicity, and to saturate the metabolism of organs. This also would contribute to a worst-case scenario assessment. Most authors have done that and quite a lot have also tested lower doses (see Table 1). The dose tested should in any case allow for a strong and consistent expression of transgene at least at the injection site. A clinically relevant dose can also be advised to compare with the high-dose results.

Study duration

As for study duration, it depends on the vector (episomal, integrating) and on the forthcoming clinical protocol. In most cases, it relies on one single injection to the patient, and therefore safety studies do not need to include a long-term follow-up of animals. Very short studies were reported (hours to a few days, see Table 1), mostly to assess peak vector levels. The rationale for duration should rely on preexisting knowledge on vector distribution and clinical protocol (see below). The route of administration is also an important parameter. Of course, the clinical route should be investigated, but some authors also add a 'worst-case' scenario route, like intravenous (i.v.) This is often useful, depending on prior knowledge of vector itself, and also depending on the pharmacological activity of the transgene. This latter issue cannot be addressed by model studies using reporter genes. Transfer of genes encoding nonstructural proteins, and particularly in the case where either they have a systemic effect, or when all their functions are not known, prompt for a dedicated biodistribution study with an i.v. group.

Biodistribution of vectors using radioactive tracers

Some teams also used radioactive tracers to study biodistribution of gene vectors. Although these techniques are not straightforward, they can bring valuable information. To investigate precisely the hepatic sequestration of adenoviral vectors after systemic administration, Zinn *et al*[25] labeled the adenovirus knob with technetium 99m (^{99m}Tc), a gamma emitter. This technique allowed to determine that one hepatocyte could bind around 17 500 Ad5-knob molecules. This study also confirmed that the spleen, kidneys and lungs collected equivalent, and 10-fold lower levels of vector than the liver, while minor amounts could be found in the stomach, intestine and muscle (10 min or 1 h after injection, mice). Plasmid DNA distribution was also studied *in vivo* using ^{99m}Tc[26] or ^{125}I.[27–29]

Biodistribution of gene transfer vector families

DNA nonintegrating vectors

Adenovirus. This vector biodistribution was the most extensively studied (35% of references in Table 1). We will first review biodistribution after an i.v. administration of AdV. Alemany *et al*[10] studied recombinant adenovirus (rAd) distribution 90 min after injection of 5×10^{10} viral particles via the tail vein in C57/Bl6 mice, using the luciferase reporter gene and quantitative PCR (lung, liver, kidney). Transgene expression was detected in all tested organs (list: see Table 1) at similar levels, except for the spleen, ovary and liver (very significantly higher levels). By qPCR, they showed copy numbers around 5000/ng DNA in the lung and kidney, but around 60 000/ng in the liver. They also performed an early kinetics of AdV (transducing units of vectors) in blood, showing a viremia that resolved to levels lower than 10 TU/μl in 50 min. Wood *et al*[8] studied AdV biodistribution 72 h after the tail vein injection of 5×10^9 PFU of a luciferase-expressing vector. Expression was found in the liver, lung, heart, kidney, ureter and bladder, with the highest level in the liver. Semiquantitative PCR results showed comparable amounts in most organs tested ($\geqslant$2500 copies/200 ng of DNA).

Kosuga *et al*[30] studied AdV distribution 7 days after tail vein injection of 10^8 PFU, by following transgene expression (beta-galactosidase and human beta-glucuronidase: GUSB, both driven by chicken beta-actin promoter) and PCR in disease model mice. In contrast to LacZ expression, which was detected mainly in the liver, GUSB was detected as well in the spleen (equivalent level) and in the heart and lung. Viral DNA was predominantly in the liver. In a systematic toxicology study of recombinant AdV in rabbits, Cichon *et al*[1] studied viremia between 10 min and 48 h. Rabbits were administered 5×10^{11} i.p./kg via the portal or ear veins. Viremia peaked around 30 min to 10^9 infectious particles/ml and decreased to 100–1000 i.p./ml at 48 h. These authors also looked at transgene expression (LacZ) and DNA (Southern blot) in several organs. Transgene expression was comparable in the liver and lung, and moderate in the kidney, while DNA was also found in the liver and lung, with minor amounts in the kidney, ovaries and muscle cells. Hackett *et al*[31] also obtained comparable results in pig, which differ quite substantially to that usually found in rodents. Indeed, after i.v. administration of vector (ear vein), 90% was found in the lung, with 55% also there after intraportal injection. Finally, Garcia-Bañuelos *et al*[7] injected 3×10^{11} vp via the iliac vein of cirrhotic rats to show a very high transduction of liver and moderate levels in the spleen and kidney (the other organs, see Table 1, tested negative).

Senoo *et al*[32] studied AdLacZ biodistribution in guinea-pig fetuses after umbilical vein injection. Through this administration, most of the transgene expression was found in the liver, and also in the kidney, intestine and placenta (nothing in the lung, muscle and pancreas), except in the vascular endothelium. This latter observation is important, because when performing biodistribution studies using PCR, it is not possible to distinguish between endothelium-trapped and tissue-transducing vector.

All vectors presented above are nonreplicating rAds. Bernt *et al*[2] studied the distribution of oncolytic conditionally replicating vectors. These vectors are derived from Ad5, but with Ad35 fibers (Ad5/35), which does not transduce liver cells. CB17 mice received two 10^{11} particles twice i.v. (on 2 consecutive days) and were killed at D4. Ad5/35 serum levels were much lower than Ad5. Ad5/35 vector was also detected at lower levels in all organs tested (liver, kidney, spleen, lung, heart), but more present in the liver in both cases. Plasma clearance by qPCR was equivalent and down to 100 vp/μl in less than 2 h.

One study reports the use of AdV through a local intravascular transfer to vessel wall, mimicking angioplasty procedure[3] in rabbit. Gene expression topology was studied 14 days after injection of 10^{10} PFU of a LacZ-expressing vector. Vector leakage induced transduction mainly of hepatocytes, and also of circulating monocytes and testis. Intravascular impact of AdV transfer was also studied by Turunen *et al*.[33]

Another interesting systemic route is intraperitoneal.[9] This route was tested because it is clinically relevant for ovarian cancer. Tumor-bearing mice were injected with 5×10^{10} vp and biodistribution was assessed after 48 h. Interestingly, the distribution was much more homogenous than after i.v. injection. Luciferase activity was found in all tested organs, with the highest levels in liver and spleen, followed by kidneys and ovaries, and then by the brain, lung and heart.

AdV distribution after intramuscular (i.m.) injection was studied in rabbits.[4] Semimembranosus was injected with 2×10^{10} PFU, and distribution was assessed by LacZ expression and RT-PCR. Some LacZ-positive cells were found in the liver, lung, testis and spleen. Using a nested PCR, viral DNA was detected in the liver, lungs and spleen 7 days after administration. The soleus muscle also tested positive at D 7. This study documents the systemic distribution of a gene transfer vector after i.m. administration.

Some authors have also tested local administration routes. Intraprostatic injection of 10^{10} vp of replication-competent or not AdVs in the prostate of male mice was followed by mating at D8 and D29, and subsequent killing of half of them.[34] This design was made to study distribution for prostate cancer therapy and germline transmission risks. Both vectors were detected in the urogenital tract and liver (300-fold more and for a longer period with the replicating vector). No germline transmission could be evidenced in the offspring ($n = 149$).

Wood *et al*[8] studied the distribution of a luciferase-expressing AdV after intravesical administration. Mice were subjected to instillation of 5×10^9 PFU of vector, directly in the bladder, after a 12 h water deprivation, and followed by a 4 h water deprivation, to ensure maximum contact. After 24 h, mice were killed and distribution assessed by semiquantitative PCR and luciferase activity. The bladder local administration resulted in local expression and localization of DNA (only one kidney and one ureter among the three studied mice tested positive). On the contrary, in another local route, intracerebral administration of AdV resulted in wide ectopic distribution[35] in the spleen, heart and lung, with transgene expression (LacZ) in the heart. This study was carried out in rats using 3×10^8 PFU of vector.

Finally, the subcutaneous route has been extensively studied in cotton rats (*Sigmodon hispidus*), a permissive species to wild-type human adenovirus 5.[15] The animals receiving the highest dose (1.8×10^{12} vp) had copy numbers around 100 per 100 ng genomic DNA in the liver, spleen, lung, kidney, brain and ovaries. At lower dose, amounts around 50–100 copies were found in the liver, spleen and lungs even at D56. The skin was of course the place where the highest quantity was found (site of injection). This study was performed for cutaneous head and neck cancer treatment. Like for i.m., subcutaneous administration of AdV results in quite a widespread distribution of vector.

Adeno-associated virus type 2 vector distribution. Ponnazhagan *et al*[36] studied systematically rAAV2 biodistribution in mouse after i.v. injection of 1×10^{10} vp (PCR+Southern blot). The vector distributed widely soon after the injection, and predominantly in the liver after 1 week (but also in the heart and muscle).

I.v. distribution of rAAV2 has mainly been studied in the application of lysosomal storage disease (MPS VII) and hemophilia (human factor IX). Distribution for this latter transgene was studied by PCR after injection via tail or portal veins of 10^{11} or 5×10^{10} vg.[37] After tail vein injection, vector DNA was detected in the liver and spleen only. After portal vein injection, the liver and spleen were also positive, but the latter at the highest dose only. Expression of transgene by RT-PCR was only detected in the liver (for PCR and RT-PCR, organ tested were the liver, lung, spleen, kidney and heart). Watson *et al*[38] studied rAAV2 distribution after beta-glucuronidase gene transfer (10^{12} particles, tail vein). Gene expression was detected in the liver, heart and muscles, and in animals treated 13 weeks before, DNA was found in the liver (other organs not tested).

rAAV2 distribution after i.m. injection was studied in cynomolgus,[39] baboons,[40] dogs,[41–43] rats,[44,41] mice and rabbits.[41] Only one extensive study of distribution was carried out.[43] In this study, several organs were tested using a nonquantitative method, in one dog (injected with 2×10^{12} vp), and rAAV2 was found only in the draining lymph node. Chan *et al*[44] also studied rAAV2 distribution in the spleen, thymus and liver and did not find it at D33. Other authors focused mainly on germline transmission risks and shedding. To address germline transmission risks, they searched for rAAV2 DNA in the genital tract of several species. Results differed quite widely. While no AAV could be found in the semen[41] or testes[43] of dogs, it was quite consistently found in gonads or epididymal effluent of mice and rats.[41] These authors also demonstrated a 48 h viremia lasting 48 h in rabbits (which is confirmed in cynomolgus by Favre[39]and in humans by Kay[45]). In rabbits, no DNA was found in the semen, while some was found in gonads.[41] These authors performed FISH analysis on testes, which showed the presence of rAAV2 DNA in the basement membrane and interstitial space.

Favre *et al*[39] also demonstrated rAAV2 DNA presence in the liver of cynomolgus at least 18 months after injection (and also injected muscle and draining lymph node). It was also present in peripheral blood mononuclear cells (PBMCs) for up to 10 months. Regarding shedding, Herzog *et al*[42] found some only 24 h after injection in dogs (1.3×10^{11}–8.5×10^{12} vp). In cynomolgus (injected with 5×10^{8}–1×10^{10} i.p.), shedding was found in all fluids for up to 6 days.[39] Lai *et al*[17] studied rAAV2 biodistribution after intrahepatic *in utero* injection in rhesus fetuses.

Plasmid DNA vectors. Plasmid DNA, formulated or not with nonviral vector systems, is the most used and oldest gene transfer system. Its safety and distribution features have been studied for more than 10 years We will review briefly here the distributions characteristics of DNA–lipid, DNA–cationic lipid and naked DNA vectors.

Following i.v. administration of DNA–lipid complexes, they localize to the liver, lung, kidney, spleen and heart early after injection,[46–50] but to much fewer tissues later (ie muscle[46]). Regarding DNA–cationic lipid complexes, they could be found in the lung, kidney, spleen and liver but not in gonads after i.v. injection of pigs and rabbits.[51] In minipigs, they were primarily found in the heart and lungs 9–11 days after injection.[52] These formulations were used in humans, with no remarkable adverse effects.[53,54] Using a sensitive technique (500 copies/μg DNA), Thomas *et al*[55] detected plasmid DNA in all organs tested up to 1 month after injection, emphasizing the importance of sensitivity and validation.

DNA in polyethylenimine complexes, administered i.v., distributed widely at early time points (15 min, 24 h), and persisted longer than naked DNA in the liver, lung and kidney (more than 10 days *versus* 2–3 days).[19] Comparison of aerosolization and i.v. injection of PEI DNA also showed a wide distribution by both routes at early times.[56]

Tissue distribution of naked plasmid DNA was also studied in mice[57,58] and rabbits[57] after i.m. and i.v. injections. After i.m. injection, plasmid was found at early time points in all highly vascularized organs, and then only in the injected muscle (up to 8 weeks). After i.v. injection, plasmid distributed in all tissues except the gonads and brain. At 4 weeks after injection, plasmid was cleared from every location, except for the lung of one of six animals. Hanke *et al*[13] found that plasmid was detectable only in injected muscle and nearby skin at D46 and D78, except for one kidney and one epididymis at day 46.

Herpes simplex 1- and 2-derived vectors. HSV-derived vectors distribution was investigated in primates[18,59,60] and mice.[60,61] After i.v. administration, DISC-hGMCSF, a gH-deleted HSV-2-based vector expressing human GM-CSF was widely distributed up to day 28, but by day 56 had disappeared from the gonads and brain and was found only in the blood and liver. Local routes (intracerebral[18,59] and intraprostatic)[60] were also investigated.

RNA integrating vectors

Moloney mouse leukemia and lentivirus-derived vectors. Most studies published so far relate to i.v. injections in mice. Pan *et al*[12] carried out a systematic VSVG-pseudotype distribution study after tail vein injection. The highest vector expression (GFP) was found in the liver, spleen and bone marrow (4–40 days after injection). These results were confirmed by qPCR. Minor

but existing quantities could also be found in the bladder, brain, kidney, heart, lung, gonads and gastrointestinal tract. It is worth noting that these authors perfused transcardially the mice for 20 min to minimize DNA contamination by blood. In a study designed to compare targeted vectors, Peng *et al*[11] also undertook a systematic distribution study with an amphotropic 4070A-pseudotype vector. The transgene used was luciferase, and the results were corroborated by PCR analysis for luciferase gene. Animals were killed 2 weeks after injection. In order of decreasing quantity, the transgene was found in the liver, spleen, heart, skeletal muscle, and not in the brain, kidney, ovary and bone marrow.

Biodistribution of an SIV-derived, VSVG-pseudotyped vector was studied by Indraccolo *et al*[62] by qPCR after intraperitoneal injection of 3×10^6 TU in a model of xenograft tumors. Analysis of the liver, spleen, lungs and kidneys showed vectors only in the liver and spleen (0.1–1 and 0.8–2% positive cells, respectively).

A study designed to compare different pseudotypes (VSVG, Mokola and Ebola) was also performed in mice for *in utero* gene therapy.[63] Vectors expressing LacZ under the CMV promoter were injected via i.m. or intrahepatic routes. Vectors were found in several organs, but at significant levels mainly in the liver, heart and muscle. Comparison of pseudotypes showed that VSVG was more efficient for hepatocytes, while Mokola and Ebola were more efficient for myocytes. Distribution after local eye instillation of an MLV vector was also documented.[64]

Germline transmission studies

In addition to the biodistribution studies reviewed above (which often include gonadal distribution assessment, especially for rAAV2), some teams reported experiments dedicated to germline risk assessment. These studies were mainly performed for AdV and rAAV2s. Regarding the former, Ye *et al*[65] injected mice with doses able to reach the gonads. They then examined the offspring ($n = 814$) by Southern blot, and could evidence to vertical transmission was evidentn. After intraprostate injection, examination of the offspring ($n = 149$) yielded similar results.[34] Using LacZ transgene, AdV expression localization in testes (sometimes positive by PCR[66] and see above) was further investigated.[66] Injection in the ventricular cavity (heart) did not allow to transduce any spermatid or mature spermatozoa (their hypothesis relied on the fact that the infection of one single stem cell should have resulted in 2048 easily detectable LacZ-positive spermatids).

Regarding rAAV2, there were two studies dedicated to germline transmission. One showed that Sertoli cells and spermatogonia-like cells showed transgene expression signals 6 months after treatment,[67] and the other that direct exposure of mature mouse spermatozoa to rAAV2 fails to lead to germ cell transduction.[68]

Discussion

The reference technique for gene transfer biodistribution is now real-time quantitative PCR with probe technology. Overall, precise specifications and guidelines are available and pending issues are discussed below. Agencies in charge of clinical trial approvals tend to prefer that gene therapy clinical trials be carried out under the same standards as for small molecules. This means that for preclinical safety studies, good laboratory practices are preferred, and in our case, validated analytical techniques.

A few years ago, the main approach was defining generic biodistribution properties of a vector class by designing studies often relying on transgene expression and mostly nonvalidated PCR techniques. These studies have progressively helped defining important features of these vectors, and have also shown that even if some important characteristics exist, an important variability is introduced by the administration route, the promoter and other key components of the construct (targeting modifications, etc). It is now currently admitted that specific biodistribution studies should be carried out prior to any clinical trial (ASGT nonclinical toxicology workshop, Arlington, VA, USA, March 13–14, 2003, see Frederickson and Pilaro[69]). The extent of these studies may differ depending on preexisting knowledge about the vector and its distribution (a new and different route of administration may necessitate a complete new study, even in a big species). The viral vector titration standardization issues, specific formulation and purification process also prompt for dedicated biodistribution and toxicology studies.

PCR technique and its validation relies on general knowledge on quantitative PCR and ICH guidelines (ICH Q2A and Q2B) and were exposed above. The only points that are sometimes discussed are the number of replicates, the necessity of an internal control and the specificity. Regarding this latter parameter, there are no recommendations. However, the use of a probe in Taqman qPCR brings a built-in high level of specificity. We would recommend, however, that amplicon be checked on an agarose gel during technique development as a quality control. To monitor precisely inhibition in each and every sample amplified on a given day, and to carry on analyses in real duplicate, we recommend triplicate amplification using one sample for spiking with an internal inhibition control (which might not necessarily be competitive). Apart from well-known inhibition problems (like due to hemoglobin), we advise to monitor this way precisely inhibition that could result from intra- or interassay variability. Organ sampling is quite well defined, the questions remaining are organ washing and perfusion while killing. Indeed, results can be modified by blood carry over because numerous vectors transduce white blood cells (rAAV2[39] and retroviruses). Regarding primers, the most important recommendation is the choice of a small amplicon.

In the course of development, the next points to consider are animal study with choice of species, duration and route. Some authors have undertaken biodistribution studies in nonhuman primates on the sole ground that they are the most 'similar' to humans.[17,40] In our opinion, species models should be used in well-defined cases like aotus or marmosets for HSV,[18,59,60] or cotton-rat for AdV.[70] Regarding biodistribution, big species can be justified as well when addressing problems that cannot find solution in smaller mammals (like special administration routes, which

cannot be modeled correctly in rodents). For instance, while the feasibility of aerosol gene transfer *in vivo* was demonstrated in mice,[71] preclinical data gathered on monkeys[72] supported a phase I clinical trial.[73]

Study duration should be decided according to vector properties and future clinical protocol. Peak vectors levels assessment might be critical when the vectorized system has properties that might render necessary patient isolation, at least for a short period. For instance, an AdV carrying a problematic transgene might pose a transient problem depending on the dose, thus prompting for a peak level biodistribution. In most cases, this will not be necessary, and a 30–90 days study would be sufficient.

Administration route is also very important. It must correctly be modeled in the distribution study. In some instances, it might necessitate a big species (see above and Sene[72]).

Results discrepancy between species or preclinical settings[31] also underscore the importance of the anatomic topography of the vein used for i.v. biodistribution testing. It is not surprising that results from a tail vein injection differ from those of a ear or portal injection, independent of the species effect. This reinforces the necessity of clinically relevant testing (same or very similar administration route).

In some cases, special routes must be tested.[74] Regarding systemic administration, caution must be taken about blood–endothelium-trapped vector, which might generate background noise in organ-specific analyses (whole blood and/or serum and PBMCs should be tested separately).

As far as generic vector classes are concerned, some important features can be underscored from published work.

All systemic routes were tested with adenovirus vectors, mostly in rodents. After i.v. injection, it is safe to consider that there is a transient viremia that lasts 48–72 h. Vector diffuses in well-irrigated organs, mainly the liver and lung,[1,31] and also in the spleen and kidneys (see above). In rodents, the liver is clearly the most important target organ (see above). There has been a discussion about the usefulness of this species for human clinical trials.[31] However, systemic administration of high-dose AdV in primates, clearly resulted in liver toxicity,[75] a result that could be predicted from studies in rodents and lagomorphs (Cichon *et al*[1] and above). It is also worth noting that the main AdV toxicity in humans was a consequence of an out-of-control immune reaction and high inflammation,[76] which was also found in baboons at the higher dose injected.[75] In rodents and lagomorphs, cytokine levels were not investigated to our knowledge, but hematological modifications were evidenced.[1] Carefully and well-designed toxicology studies even in rodents, relying on sound biodistribution, can therefore help predicting vector's fate in humans, as in small molecules. Interestingly, the administration of AdV i.m. or subcutaneously also showed systemic distribution, in the same target organs as by the i.v. route, but to much lower levels.[4,15]

Recombinant AAV distribution was studied early by Ponnazhagan,[36] showing results quite similar to AdV distribution. An important point with this vector is that it persists for very long periods in PBMCs (up to 10 months[39]) and in serum (up to 6 days[39]). rAAV, as AdV, also induces a transient viremia.[41,39,45] The controversial issue of rAAV2 in sperm[76] has prompted dedicated studies, which also looked at shedding (see above).

Regarding plasmid DNA, its distribution depends on its formulation. Generic ones are reviewed above. It is clear that each change in complexes or liposomes used for DNA transport and intake in cells should be investigated with regard to its impact on distribution. It seems clear that plasmid distributes widely as other gene transfer media shortly after injection, and can then persist for weeks at and near the injection site. Quite extensive research has also been devoted to formulations for aerosolization.[77–80]

The MLV and lentivirus distribution studies pose the problem of biodistribution of *ex vivo* modified cells. Indeed, these vectors are mainly used in that kind of clinical setting. Studies reviewed above focus on *in vivo* delivery of the vector. These kind of studies can bring valuable information, but they do not parallel what will be done in the clinic. Even as worst-case investigation, they are questionable, because the cell intake will be highly divergent between a lentivirus and an *ex vivo* lentivirus-transduced cell. The debate is not settled, and more extensive investigations need to be performed.

Generic dedicated germline transmission studies have been carried out. The most important point to consider is their relevance compared to what can happen in the clinical setting. Depending on the route of administration, the direct exposure of mature spermatozoa might not be relevant to a clinical risk of spermatogonia transduction,[68] which was expected in rAAV2 systemic administration.[66]

By the results it yields in terms of target organs for toxicity, bioavailability and risk assessment, biodistribution study paves the way for nonclinical toxicology. It is often the first step in clinical conditions (same vector, correctly modeled route, model duration), and thus a pivotal study in preclinical gene transfer development and toxicology. It might also be a first step towards innate evaluation by cytokines measurements on serum samples.

There are now some contract research organizations (CROs) that can carry out biodistribution studies under GLPs, with validated and good-performance techniques. While this review showed results published by academic labs (sometimes including biodistribution studies subcontracted to CROs), a lot of unpublished results produced by CROs have been produced. The field of gene transfer preclinical studies is progressively shifting to the private sector, which is good news for the industry.

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