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Genetic heterogeneity in response to adenovirus gene therapy

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Abstract

Background: After intravenous delivery of the adenoviral vector into rats or mice, 95–99% of the encoded protein is produced in the hepatocytes. We observed, as have others, that the early expression levels of the vector encoded protein vary, greatly, within a species, from one animal strain to another. This study was initiated to determine the molecular mechanism causing the difference: hepatic transfection, transcription or translation. For this purpose different doses of Ad5 luciferase and Ad5 LacZ were intravenously injected into Brown Norway rats and Wag/Rij rats, two strains that differ by a factor of 10 in encoded protein levels. The proportion of LacZ positive hepatocytes, the adenoviral DNA, specific transgenic RNA and luciferase protein were compared in the two strains.

Results: The number of transduced hepatocytes and the amounts of Ad5 DNA in the livers was similar in both strains, whereas the Brown Norway rats produced 8 to 10 times more of both vector encoded proteins and of transgene mRNA than the Wag/Rij rats.

Conclusions: It is concluded that the difference between strains in vector encoded protein expression is due to different transcriptional events. No evidence was obtained to suggest that the differences are related to liver damage influenced by vector toxicity or immune reactions.

Background

In the course of studies involving intravenous adenoviral gene transfer, we observed a 10 fold difference in plasma levels of the encoded proteins between two of the rat strains employed: the Wag /Rij and the Brown Norway. These differences were observed for several vector encoded proteins as soon as 2 days after gene delivery and were maintained for one month, after which the levels declined slowly to background. Other investigators described differences of the same order of magnitude between mouse strains in expression of transgenes encoding α1-antitrypsin [1], interleukin-12 [2], and human endostatin [3]. These differences in transgene expression between inbred species may have clinical implications, as differences between inbred strains are considered to reflect differences between individuals of an outbred species.

Consequently, the therapeutic efficiency of a transgene could drastically vary from one patient to another. So far, a satisfactory explanation for such strain differences has not been provided. Most authors have attributed the difference of plasma levels of vector encoded protein to immune reactions against the protein or against the vector [4,5]. However, the kinetics of the vector encoded proteins in the plasma do not support regulation by an adaptive immune reaction, as the strain difference is manifest

as early as 2 days after administration of the vector. Innate humoral immunity, e.g. the presence of neutralising antibodies against the vector can also be excluded as the cause of the difference because such antibodies are not present in naïve rats. An innate cellular immune reaction directed against the infected liver cells is also an unlikely explanation because plasma transaminase levels [6] – a marker of hepatolysis – though somewhat elevated, are not different in the two strains.

The main organ infected by an adenovirus following iv injection is the liver [7,8]. Others and we find that the liver is producing 95–99% of the vector encoded protein after intravenous delivery of recombinant adenoviral vectors. The global process from liver cell infection to protein production is commonly described as adenoviral permissiveness of the liver. In this study we investigated which one of these three processes (transfection, transcription, translation) is pivotal for the strain difference in transgene expression.

Secreted vector encoded proteins are not suitable to interpret the early manifestation of transgene expression, as plasma levels are highly dependent on the pharmacokinetic properties of the encoded proteins. Therefore, we employed adenoviral encoded marker genes, the intracellular Luciferase (Luc) and the nuclear β-Galactosidase (LacZ). The luciferase activity of the whole liver is a measure of vector encoded protein expression in that organ while the LacZ staining of hepatocytes provides information on the proportion of liver cells infected. Furthermore, we determined the amount of Ad5 DNA and of transgene mRNA in the liver as measures of the adenoviral infection of hepatic cells and of transgene transcription respectively.

Results

Intravenous injection of Ad5 vectors in different rat strains results in different levels of early transgene expression

Intravenous administration of a fixed dose of Ad5 vector either expressing the human endostatin gene, or the gene for mhATF-BPTI resulted in clear 10-fold difference in the plasma levels of both proteins between Brown Norway and Wag/Rij rats (Fig. 1 and 2). This difference was detected as soon as 3 days after the vector administration and persisted during the first 6 weeks, subsequently the plasma levels of both proteins slowly decreased to undetectable levels in 2 to 3 months. To investigate whether the livers of the 2 rat strains differ in their permissiveness for Ad5 or if this phenomenon could be explained by differences in the hepatocyte secretion capacity, we administrated Ad5 vector carrying the gene for Luciferase (Ad5 Adapt Luc), a protein that is not secreted.

Measurements of Luciferase transgene activity in various organs, following iv administration of the vector, confirmed the observations from other research groups [7,8] that in rodents 95–99% of virus infection and subsequent transgene expression occurs in the liver (Table 1).

Next, we determined luciferase activity in the liver of Brown Norway and Wag/Rij rats 2 days after intravenous injection of 10^9 iu, 3.10^9 iu, or 10^{10}infectious units (iu) of Ad5 Adapt Luc. The results depicted in the Fig. 3 seem to reflect those observed with ATF-BPTI and endostatin naming a 10-fold difference between Wag/Rij and Brown Norway rats. These experiments with the non-secreted luciferase vector encoded protein confirmed that the two rat strains have a very different liver response to the recombinant adenovirus. Since the levels of mhATF-BPTI and endostatin reached a plateau about 7 days after vector delivery both in the Brown Norway and in the Wag/Rij rats, we also determined the luciferase activity in the liver 7 days post intravenous administration of the Ad5 Adapt Luc. As shown in figure 4, roughly the same values were obtained at the 2 time points, which reflect the kinetics of the plasma concentrations of the secreted proteins ATF-BPTI and endostatine.

β-Galactosidase expression in hepatocytes from Brown Norway and Wag/Rij rats

To explore whether differences in the number of transduced hepatocytes could account for the observed differences in transgene expression levels, we injected different doses (10^9 iu, 3.10^9 iu and 10^{10} iu) of Ad5 Adapt LacZ into the tail vein of the rats. Histological inspection of the stained sections allowed an estimation of the proportion of transduced liver cells. Animals were sacrificed after 2 days, liver sections were prepared and stained with X-gal. The liver sections revealed an inhomogeneous distribution of transduced cells. In both rat strains hepatocytes were the sole cell type expressing the β-Galactosidase. Both the number of stained cells and the staining intensity was highest near the interlobular (portal) vein and declined gradually towards the centro lobular area (Fig. 5). The percentage of blue nuclei was roughly proportional to the amount of infectious units administered and did not differ significantly between the two strains (Table 2). However, overall, the intensity of the nuclear LacZ stain was 8 to 9 times higher in the Brown Norway than in Wag/Rij liver sections as determined by computer-assisted analysis of the histological section (Fig. 6). These findings suggest that although the protein production is significantly higher in the Brown Norway rats, the number of infected cells is roughly similar for both rat strains. Still the number of infectious particles per hepatocytes might be higher in the Brown Norway rats. However the number of iu can never be 10 time higher because that would require at least 10 × the number of hepatocytes ($1.8\ 10^9$) = $1.8\ 10^{10}$, and the dose administered was 10^{10} iu.

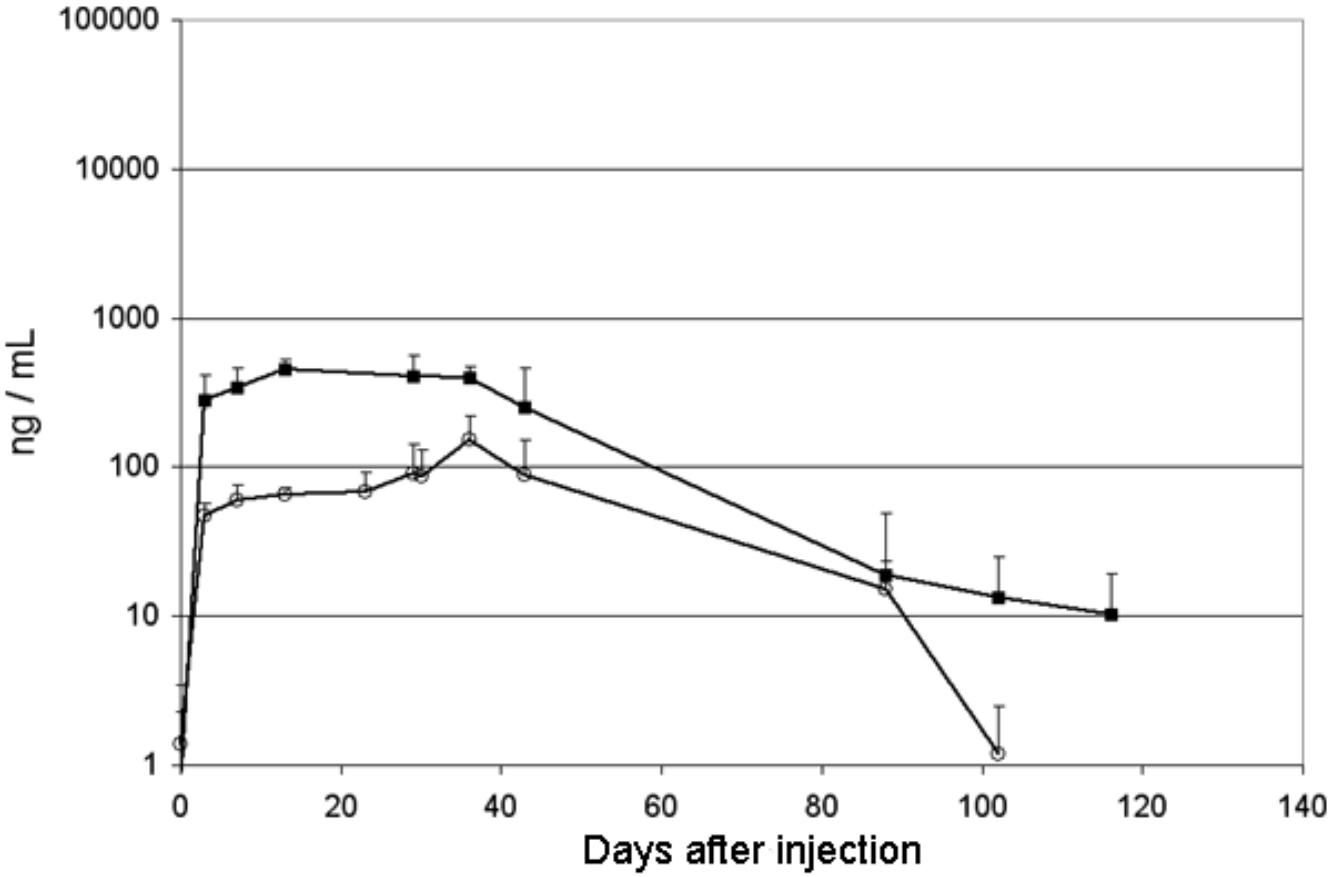

Figure 1
Plasma levels of ATF-BPTI in Brown Norway (black squares) and in Wag/Rij (open circle) after iv injection of 10^{10} iu Ad5 Adapt mhATF-BPTI. Ten animals per group, data are expressed as mean ± SD.

The number of recombinant adenovirus copies in the liver

Real-time PCR was employed to quantify the copy number of the adenoviral genome in the hepatocytes of rats from the previously described experiments that received graded doses of Ad5 Adapt Luc, or of Ad5 Adapt LacZ. As listed in Table 3, the number of adenoviral copies per liver was similar in the Brown Norway and Wag/Rij rats for both vectors. Roughly, there is a linear relation between the Ad5 DNA detected and the dose of adenoviral vector administered. In the case of LacZ, the deviation from the linearity is not statistically significant.

These results shows that recombinant Ad5 infect hepatocytes of both rat strains with similar efficiencies. Thus, the observed differences in levels of vector encoded protein must be due to differences in transcription (CMV-promoter activity), RNA stability, RNA translation, or post translational processes.

Liver damage

Considering that the local inflammation of the liver may significantly increase the synthesis of certain proteins, it could be that the difference in transgene expression observed is a consequence of different inflammatory reactions in the two strains. To eliminate the possibility that the difference found in the Brown Norway rats in encoded protein is due to a global increase in protein synthesis, the total amount of liver protein was determined after different vector doses and time points. It was found to be similar in both strains and not significantly elevated from the basal line (Table 5).

Adenovirus particles in apoptotic hepatocytes or phagocytosed by immune cells would not contribute to the luciferase production. However, to exclude that a massive hepatitis or a severe inflammatory infiltrate in the liver is causing the difference of luciferase expression, the inflammatory infiltration and pathological changes were

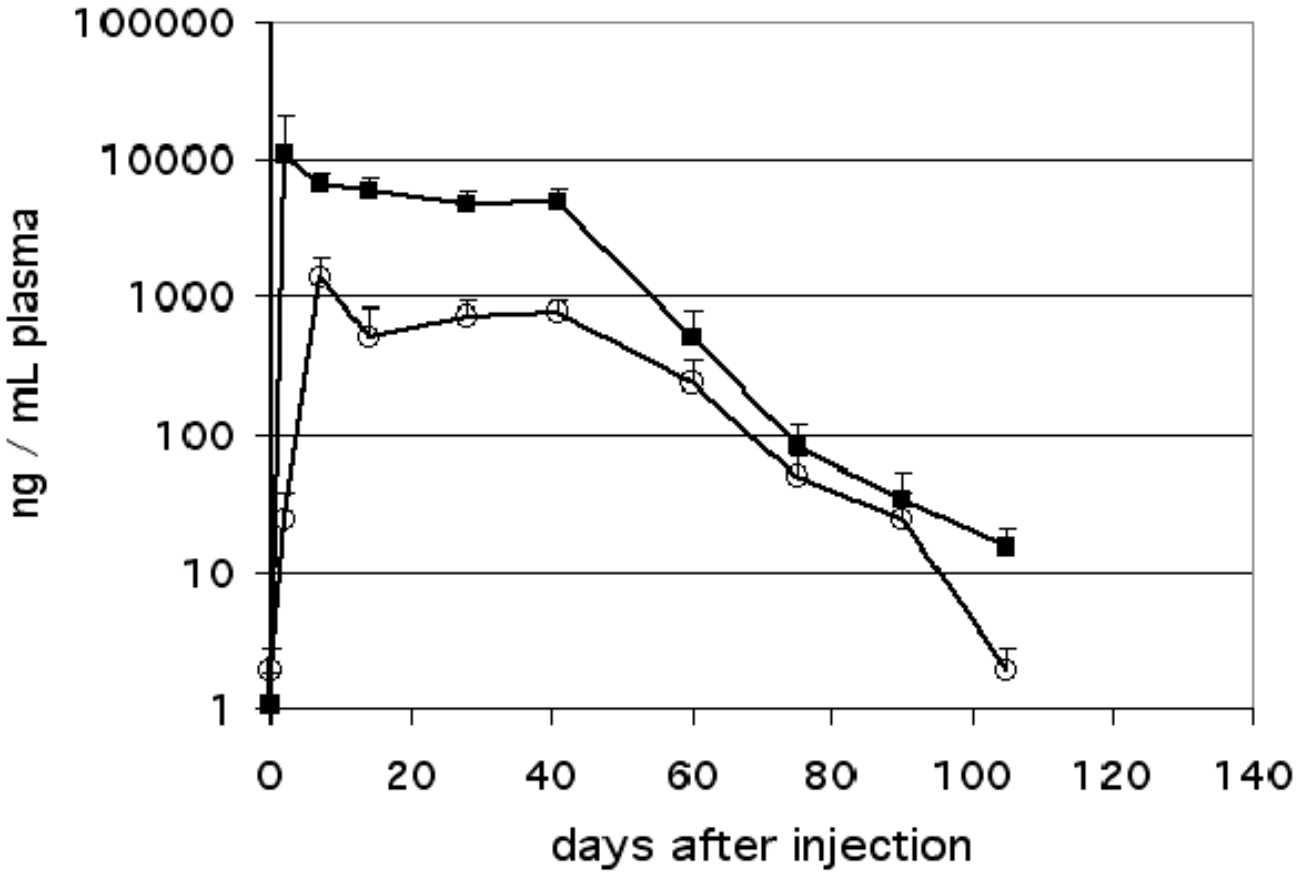

Figure 2
Plasma levels of endostatin in Brown Norway (black squares) and in Wag/Rij (open circle), after iv injection of 10^{10} iu Ad5 Adapt hEndostatin. Ten animals per group, data are expressed as mean ± SD.

Table 1: Percentage of total luciferase activity per organ measured in the Brown Norway and Wag/Rij rats 2 days after intravenous administration of 3.10^9 iu of Ad.Adapt.luc virus. The luciferase activity is expressed in RLU / mg protein. Data are expressed as mean ± SD. Fresh organs were weighted and the mean value is expressed in grams / 100 grams body weight. The muscle mass was estimated from the human situation as 40% of total body mass. The bone marrow was previously measured.

Organs	**Weight** (g / 100 g)	**Brown Norway** (n = 14)	**Wag/Rij** (n = 6)
Liver	3.4	**97.91 ± 2.35**	**96.88 ± 4.76**
Spleen	0.3	1.93 ± 2.05	1.99 ± 1.52
Heart	0.5	0.072 ± 0.04	0.06 ± 0.05
Lung	0.8	0.037 ± 0.04	0.04 ± 0.005
Kidney	1.1	0.001 ± 0.008	0.03 ± 0.02
Muscle	40	0.005 ± 0.06	0.89 ± 0.92
Brain	1	0.003 ± 0.006	Not done
Bone Marrow	1	0.028 ± 0.01	0.11 ± 0.23

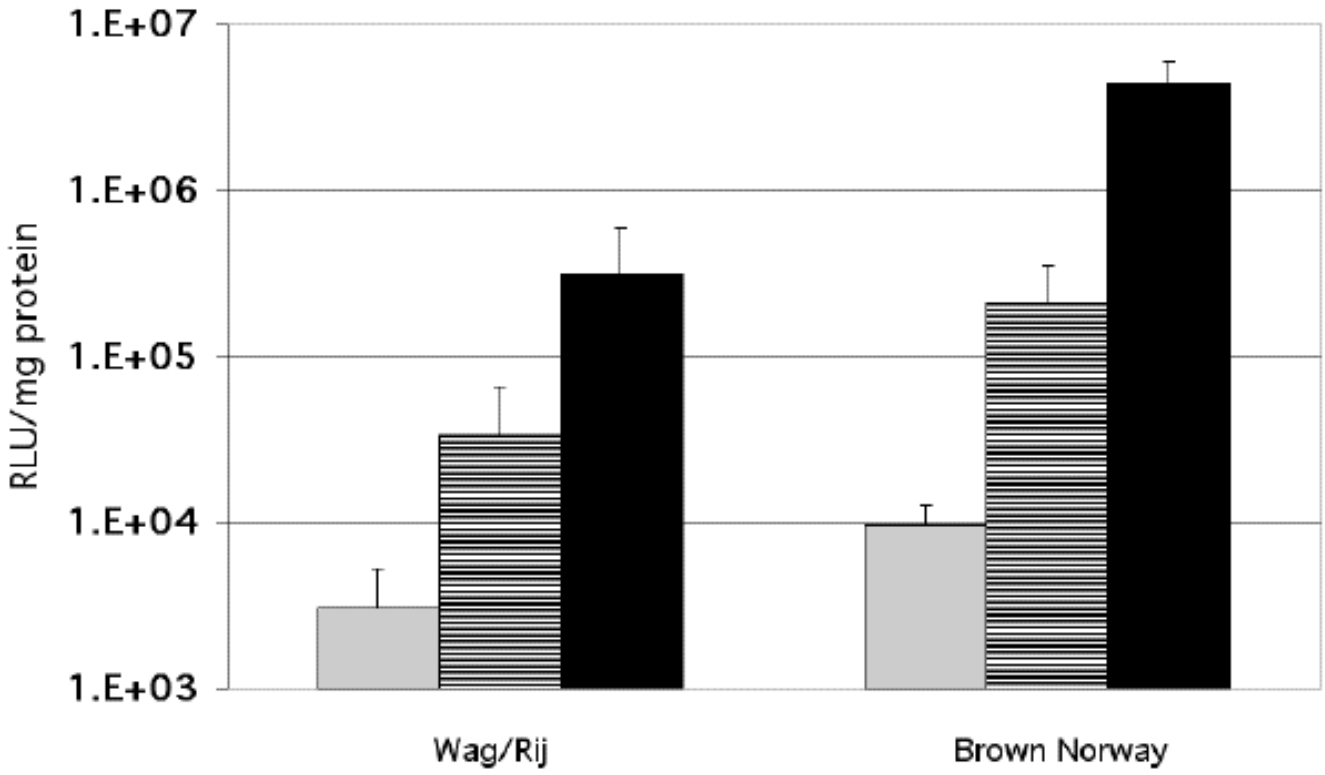

Figure 3
Luciferase expression in the liver of the Wag/Rij and Brown Norway rats. A: luciferase activity determined 48 hours after iv injection of 10^9 iu (gray bars, n = 6), 3 10^9 iu (striped bars, n = 6), and 10^{10} iu (black bars, n = 12) Ad5 Adapt Luc. Data are expressed as mean ± SD.

determined in the liver of the two rat strains (Table 5). The damage scoring shows that there is no difference of toxicity or infiltration 2 days after vector delivery and that the difference in transgene expression persisted even when a low dose of adenovirus that caused hardly any inflammation was injected. At 7 days after the injection, the Brown Norway rats showed a significant higher liver damage and infiltration than the Wag/Rij rats. However, this deviation of the toxicity between the two strains was not accompanied by an increase in the difference of transgene expression. Seemingly, the difference in transgene expression, seen 2 days as well as 7 days after vector administration, is due to processes that occur after the penetration of the virus into the cells.

Northern Blotting of the Luciferase transcript

To investigate whether an intrinsic difference exists in transcription efficiency or in translation efficiency, the amount of the luciferase mRNA present in the liver of rats was determined after administration of 10^{10} iu Ad5 Adapt Luc intravenously. As depicted in Fig. 7, the northern blot of the liver RNA shows about 10 times more luciferase mRNA in the liver of the Brown Norway rats than in the Wag / Rij rats. These findings leave open only two options to explain the difference in protein levels observed, namely an intrinsic difference in transcription efficiency (under the CMV promoter) or a difference in RNA stability.

Discussion

It has been suggested that, after iv administration, low dose intravenous adenoviral vectors are taken up by Kupffer cells and degraded quickly within a few hours [1,9] whereas higher doses saturate these cells and the surplus productively transduce hepatocytes. Therefore, we used doses (10^9 iu – 10^{10} iu) that are in the linear part of the dose response curve and well above the threshold effect. In both rat strains, 2 days after intravenous delivery of the vector, the β-Galactosidase was observed only in the

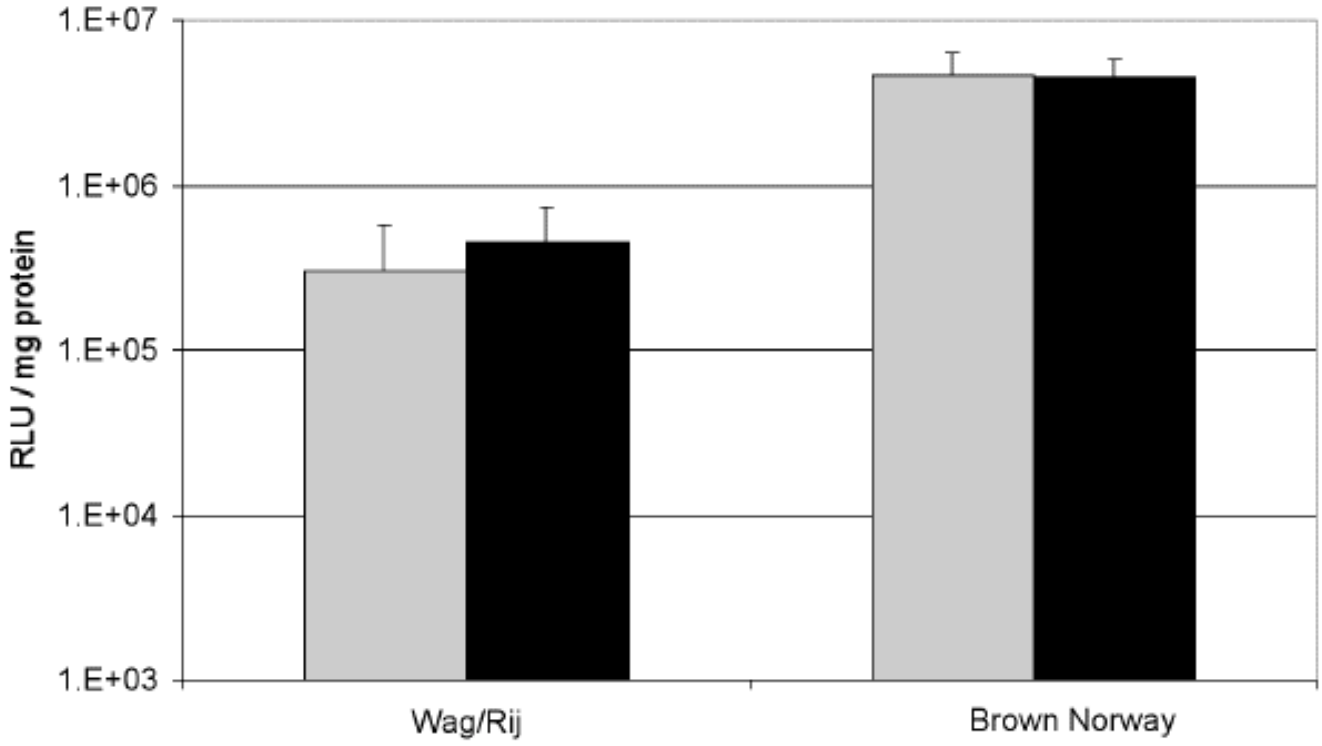

Figure 4
Luciferase expression in the liver of different rat strains after iv injection of 10^{10} iu Ad5 Adapt Luc. The luciferase expression was determined two days (gray bars) and seven days (black bars) after virus injection. Determination after 2 or 7 days reveals a 10-fold difference (p = 0.0001, Scheffé post-hoc test.) in luciferase activity between the Wag/Rij and the Brown Norway. Six animals per group, data are expressed as mean ± SD.

hepatocytes. Consequently, it may be concluded that the bulk of the adenoviral genomes in the liver is localized in the hepatocytes.

After correction for the extraction efficiency of DNA, an average of 2.6 10^9 copies of vector genome were detected in the livers following administration of 10^{10} iu Ad5 Adapt LacZ. At this dose 75% of the hepatocytes stained positive for β-Galactosidase. It is estimated that the liver of a 350 g rat contains about 1.8 10^9 hepatocytes [10]. Thus the average number of infectious units per hepatocyte is about 1.5. It is assumed that the infection of hepatocytes by the adenoviruses is a random event following the Poisson distribution. Then, this event will occur at least one time in 75% of the cases when the expected number of occurrences is 1.5. As about 75% of the hepatocytes stained blue following injection of 10^{10} iu, an hepatocyte will stain blue if it has incorporated one copy of the vector. Poisson probability calculations show also that under these conditions the infection occurs twice in 40% and three times in 20% of the hepatocytes. This variation in the number of adenoviral copies per cell can explain the variation of the staining intensity per cell within one liver, from light to dark blue.

In each of the vectors used in this study the transgene was under the control of the CMV immediate early promoter. It was determined, by Norther Blots, that the amount of transgenic mRNA was also different in both rat strains. Thus, the difference in vector encoded protein production found is due to difference in transgene transcription or difference in RNA stability.

As expected, a difference in protein levels was observed following 3 different doses of the vector 10^9, 3 10^9 and 10^{10} iu. However, the lower the dose, the smaller is the difference of protein expression between the two rat strains. This might be explained by a saturation of the transgene expression machinery in the Wag / Rij hepatocytes at the higher doses.

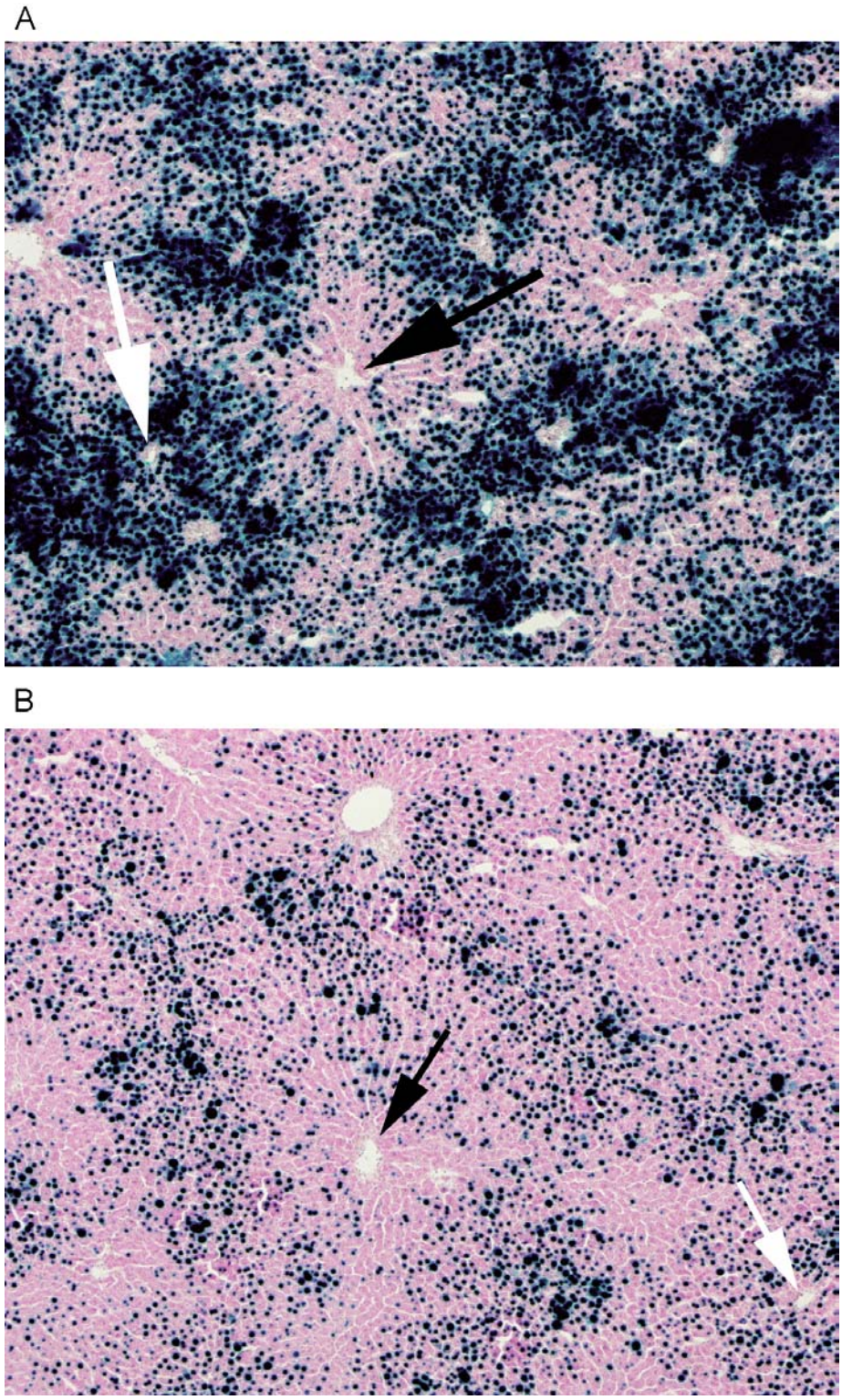

Figure 5
The "porto-cava" gradient of the transgene expression viewed on a 50 × magnification field of liver section stained for X-Gal and counterstained with red. The livers were collected 2 days after iv injection of 10^{10} iu Ad5 Adapt LacZ A: Brown Norway rat. B: Wag/Rij rat. The white arrow points to a interlobular (portal) vein. The black arrow points to a central vein in the centro-lobular area.

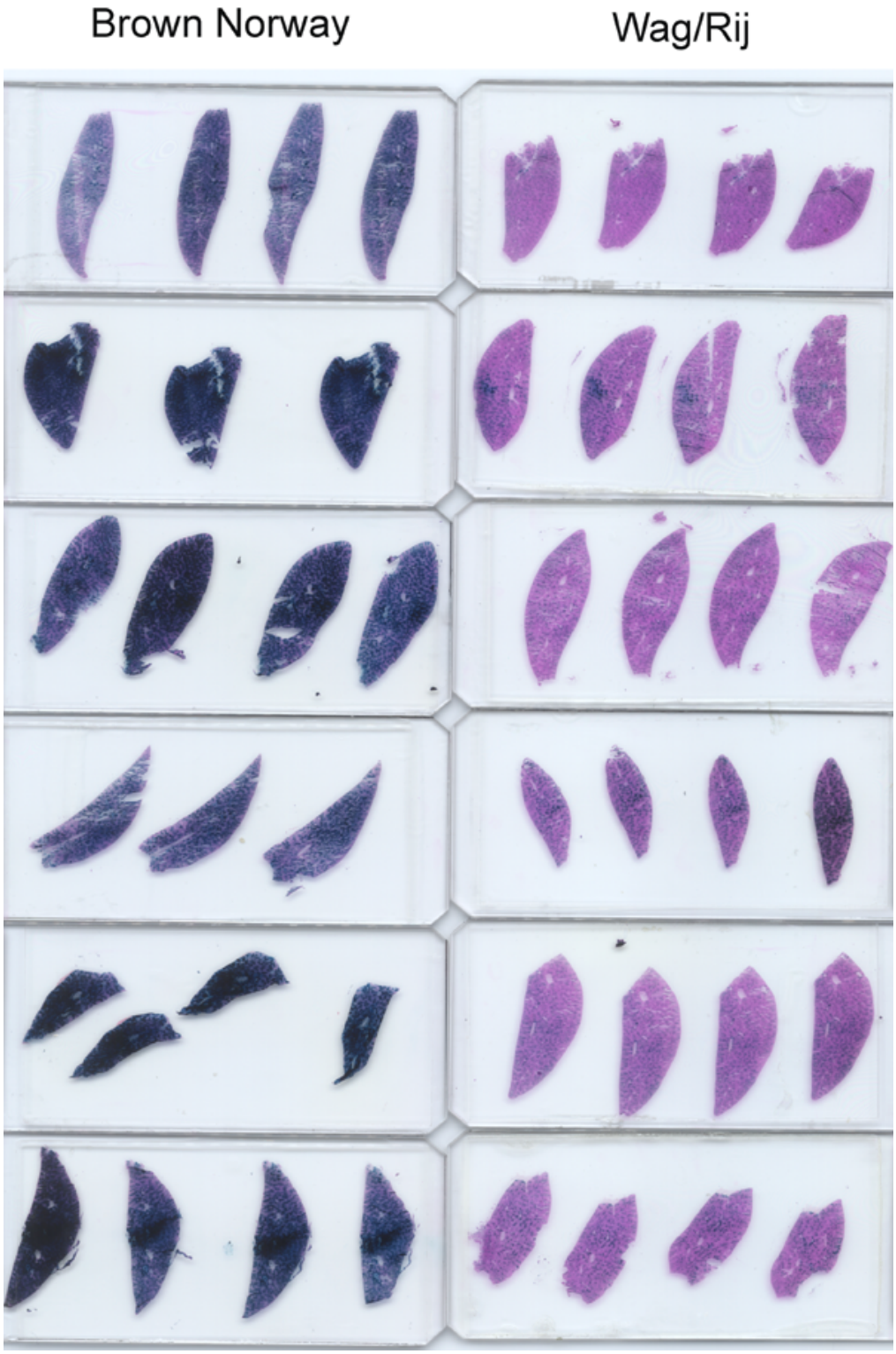

Figure 6
Comparison of histological sections of the liver of Brown Norway and Wag/Rij rats 2 days after iv injection of 10^{10} iu Ad5 Adapt LacZ. The staining was carried out on 10-μm thick frozen sections. Four sections per slide, each slides represent one rat.

Table 2: Percentage of hepatocytes expressing β-Galactosidase after iv injection of Ad5 Adapt LacZ. The blue nuclei were counted on a 200 × magnification field of a liver section. Four fields per section, 4 sections per animal. Data are expressed as mean ± SD

		Percentage of blue hepatocytes			
		Wag/Rij		Brown Norway	
Dose	**N**	Portal area	Centro-lobular area	Portal area	Centro-lobular area
10^{10} iu	**3**	82 ± 5.1	69 ± 4.6	92 ± 4.5	74 ± 3.0
3.10^{9} iu	**3**	37 ± 11.3	32 ± 11.3	57 ± 20.5	49 ± 15.6
10^{9} iu	**6**	10 ± 0.7	8 ± 0.7	19 ± 4.6	14 ± 2.5

Table 3: Number of Adenoviral DNA copies per liver determined by duplex real time PCR. This table refers to the animals depicted in Fig. 3 and in Table 2. The number of DNA copies was determined from a small weighted mass of liver and then extrapolated to the total liver mass. Data are expressed as mean ± SD

			Adenoviral copies ($\times 10^7$) / liver	
	Dose	**N**	**Wag/Rij**	**Brown Norway**
Ad5 Adapt Luc	**10^{10} iu**	**6**	160 ± 80	130 ± 50
	3.10^{9} iu	**6**	17 ± 11	19 ± 12
Ad5 Adapt LacZ	**10^{10} iu**	**6**	159 ± 50	161 ± 51
	3.10^{9} iu	**3**	45 ± 12	28 ± 8
	10^{9} iu	**3**	5.2 ± 0.7	9.4 ± 3

Table 4: Criteria for grading the liver lesions The pathological changes were graded on 3 transversal sections of the right liver lobe. The total damage score is a compilation of scores of apoptosis, vacuolar changes, nuclear condensation, anisokaryosis, megalocytosis, mitosis, and inflammation. All scores are ranging from 0 to 3. The vacuolar change was defined as clear cytoplasm for more the 90% of the nuclear circumference.

	Score			
	0	**1**	**2**	**3**
Apoptosis Number of apoptotic cells / bodies in 10 random 20 × magnification fields	0–1	<5	5–13	>13
Vacuolar change Percentage of hepatocytes affected.	No or sporadic	10 %	10–50%	> 50%
Condensed nuclei Percentage of hepatocytes affected.	No or sporadic	10 %	10–50%	> 50%
Anisokaryosis	Normal variation.	5–10% of the nuclei with 2 time the normal size	10–30% of the nuclei with 2 time the normal size	10–30% of the nuclei with 3 time the normal size
Megalocytosis	No or sporadic	3–10 / 2 sections	> 10 / 2 sections	> 30% of hepatocytes
Mitosis Number of mitosis in 10 random 20 × magnification fields	0–1	<5	5–13	>13
Inflammation I Inflammation in the Periportal Space (PS)	Normal background	Few PS with mild inflam.	Many PS with mild inflam. Or Few PS with moderate inflam	Many PS with moderate inflam. Or Few PS with severe inflam.
Inflammation II Diffuse or multifocal inflammation in sinusoid and central veins	Normal background	Increase of inflammatory cells	Aggregates of inflammatory cells	Aggregates of inflammatory cells obscuring the liver architecture

Table 5: Liver damage caused by adenoviruses. The liver lesions were assessed, as described in table 4, two and seven days after the intravenous injection of the adenoviral vector. The total liver inflammation and damage scores range from 0–6 and 0–24 respectively. The transaminase (ALAT) was measured in the serum on day 2 and 7. The normal value of ALAT, measured in ten healthy animals, is 59 +/-19 IU/l. 6 animals per group. The values indicate the mean ± sd

	High dose (10^{10} iu)		**Low dose** (10^9 iu)	
	Brown Norway	**Wag / Rij**	**Brown Norway**	**Wag / Rij**
Total liver inflammation				
• Day 2	0.50 ± 0.31	0.41 ± 0.37	0.58 ± 0.20	0.41 ± 0.20
• Day 7	3 ± 1.0	1.3 ± 0.4	1.08 ± 0.49	0.66 ± 0.25
Total liver damage				
• Day 2	2.6 ± 0.5	2.1 ± 0.7	0.8 ± 0.4	0.5 ± 0.2
• Day 7	14.5 ± 2.6	9.3 ± 1.1	4.9 ± 0.8	2.7 ± 0.7
ALAT (IU/l)				
• Day 2	63 ± 15	58 ± 16	44 ± 22	55 ± 19
• Day 7	218 ± 33	104 ± 17	86 ± 27	86 ± 27
γGT (IU /l) day 2	4.5 ± 0.6	4.8 ± 0.5	Not done	Not done
LDH (IU /l) day 2	837 ± 249	741 ± 356	Not done	Not done
INF-γ (pg / ml) day 2	60 ± 14	65 ± 18	Not done	Not done
Liver proteins (mg/g tissue)				
• Day 2	150 ± 21	150 ± 33	110 ± 21	120 ± 18
• Day 7	167 ± 32	177 ± 31	130 ± 42	136 ± 25
Luciferase (10^4 RLU/mg protein)				
• Day 2	441 ± 158	31 ± 27	1.2 ± 0.3	0.25 ± 0.12
• Day 7	451 ± 128	44 ± 29	1.5 ± 0.5	0.27 ± 0.17

The different protein expression in Wag / Rij and Brown Norway rats was observed with 4 different transgenes (Luciferase, β-Galactosidase, endostatin and mhATF-BPTI) and at several different doses. Our experiments localised the mechanism of this difference at the transcriptional level and disqualified the hypothesis based on immunological induced death of the infected cells. Nevertheless, the differences of transcription efficiency observed between the two rat strains might be due to different cytokine production following the adenoviral infection. It was shown that the adenoviral infection of the liver activates the innate immunity with increase NK cells and cytokines production (INF-γ, TNF-α, IL-12, IL-6) [11–14]. Further, differences of this activation between mouse strains were associated with variation in adenovirus transgene expression [11,13]. However it was demonstrated that no activation of the innate immunity and that no difference of transgene expression could be observed before the seventh day after the adenoviral administration [13]. Furthermore the decrease of transgene expression after activation of the innate immunity following i.v. administration of adenovirus was due to decrease of the number of infected cells and a corresponding diminution of the viral DNA in the liver [15]. Our results do not show such results. However, in such perspective we measured the serum level of INF-γ in both rat strains 2 days after the vector administration and no difference could be observed (60 ± 14 pg / ml in the Wag/Rij rats versus 65 ± 18 pg / ml in the Brown Norway rats). Nevertheless, difference of intracellular cytokine response to the adenoviral infection might explain the difference in transcription efficiency.

In general, differences between inbred strains are considered to reflect differences between individual members of outbred species. If the variation observed in the rat strains described here would hold for individual humans, the therapeutic efficiency of gene transfer could drastically vary from one patient to another after systemic administration of adenoviral vectors.

Methods

Animals

Pathogen-free inbred male Wag/Rij and Brown Norway rats, weighing 300 to 350 gr were purchased from Harlan, The Netherlands. All animals were fed ad libitum with laboratory chow and water and were kept under standard laboratory conditions. For assay of plasma transaminase (ALT), hEndostatin, and mhATF-BPTI, rats were anaesthetised with isoflurane and bled by tail vein puncture and the blood was collected in EDTA tubes. All animal procedures were performed in accordance with the official guidelines after obtaining permission of the animal welfare committee. Measurements of ALT were performed according to standard clinical procedures.

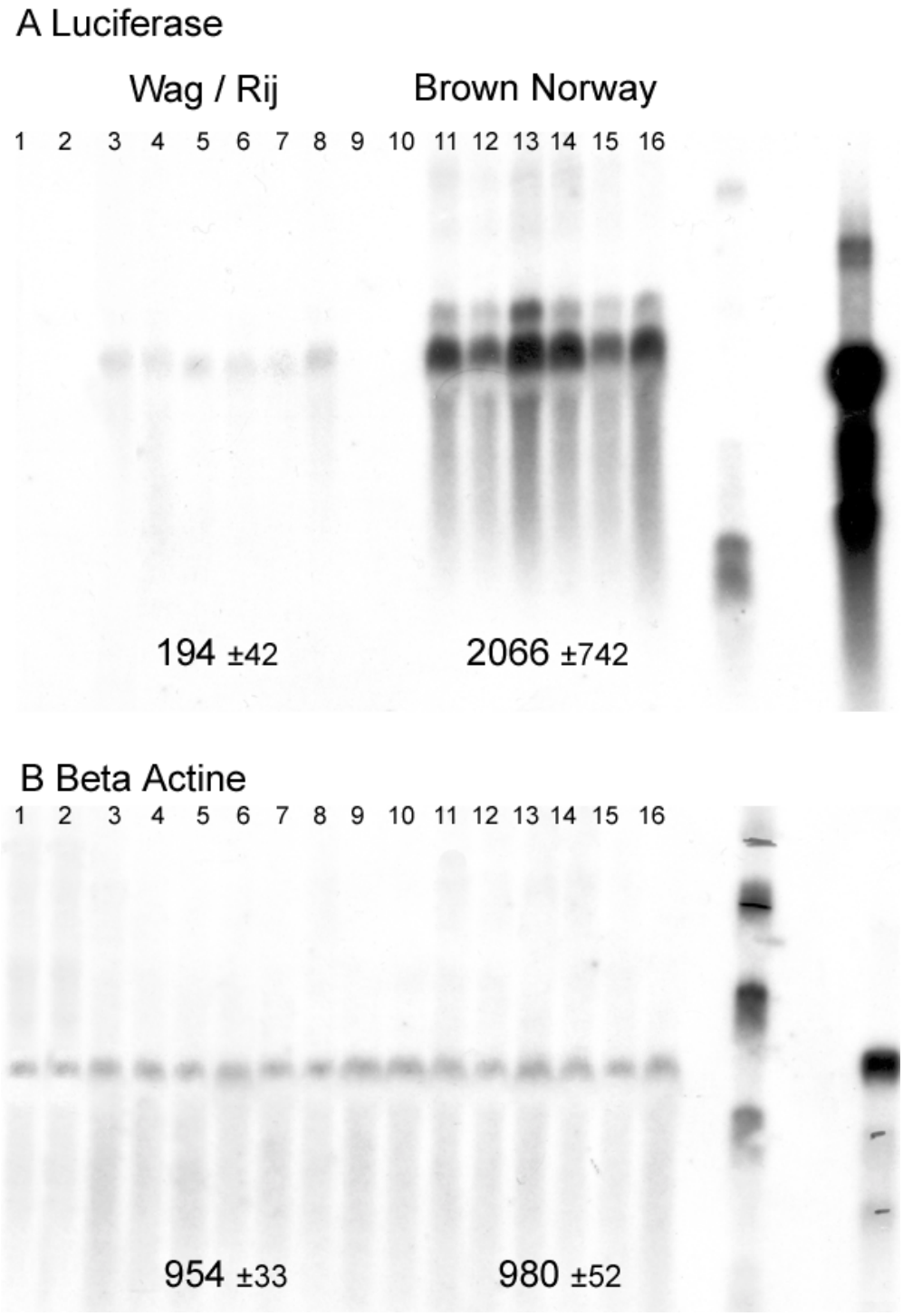

Figure 7
Northern Blots. Northern blot of the total RNA extracted from the liver of both rat strains. The hybridisation was done with a radioactive probe specific for the luciferase mRNA and then with a probe specific for the β-Actine mRNA. Lane 1–8: Wag/Rij rats, lane 1–2 animals injected with Ad5 Adapt Empty, lane 3–8: animals injected with Ad5 Adapt Luc. Lane 9–16: Brown Norway rats, lane 9–10 animals injected with Ad5 Adapt Empty, lane 11–16: animals injected with Ad5 Adapt Luc. The quantification of the signals with the phosphorimager shows that there is no difference in the quantity of β-actine transcript whereas there is about 10 times more luciferase transcripts in the liver of the Brown Norway rats than in the Wag/Rij rats. Means values +/- sd expressed in arbitrary units are depicted on the gel.

Adenoviral vectors

Recombinant adenovirus vectors were generated in PER.C6™ cells by homologous recombination between an adapter plasmid (pAdapt) and the E1 deleted Ad 5 DNA plasmid as described elsewhere [16]. The expression cassette contains a CMV promoter and SV40 poly A signal. As a result of the absence of sequence overlap between the Adapt plasmid and the Ad5 E1 sequences integrated into the genome of PER.C6, the vector stocks used in this study did not contain replicative competent adenovirus (RCA) [17].

The Ad5 Adapt mhAB encodes for a murinised form of the human ATF-BPTI (mhAB)[18]. In this construct the mhATF-BPTI, is preceded by the native secretion signal peptide of the human urokinase. The human endostatin coding sequence (InvivoGen, CA, USA) was cloned in the Ad5 Adapt shuttle vector. The encoded endostatin corresponds to the 183 residue of the human endostatin described by O'Reilly et al (1997) with an intact N-terminus (HSHRDFQ...), preceded by the secretion signal peptide of the human IL-2. The Ad.Adapt.Luc and the Ad.Adapt.LacZ are recombinant adenoviral vectors in which the E3 region of Ad5 is retained and the gene of interest (luciferase or LacZ) replaces the E1 region. The Ad5 Adapt empty is identical to Ad5 Adapt mhAB except that it does not encode any transgene.

All vectors were produced on PER.C6™ using standard procedures [17]. Infectious units (iu)/ml were determined by end point cytopathogenic effect (CPE) assay on 911 cells [19]. Viral particles were determined by HPLC [20]. The particle to infectious unit ratio was always lower than 10. A single batch per vector was used in all experiments.

Liver lysate and Luciferase activity assay

Rats were sacrificed by an overdose of isoflurane and whole organs were dissected out, frozen in liquid nitrogen and stored at -80°C. Organs were homogenised in phosphate buffered saline pH 7.8 using a blender. To lyse the cells, DTT (SIGMA, The Netherlands) (1 mM) and Triton x-100 (0.1%) (Merck, The Netherlands) were added. After centrifugation at 10,000 rpm for 10 min, 20 μl of the supernatant was added to 100 μl of luciferase assay substrate (Promega, The Netherlands). Relative light units (RLU) were determined for 30 s using a luminometer (Lumat 951, Wallac, Belgium). The amount of protein in the extracts was determined using a commercial kit (Bio-Rad laboratories, The Netherlands) based on the Coomasie brilliant blue G250 binding assay developed by Bradford [21]. The level of luciferase activity in the tissue homogenates was expressed in RLU / mg protein. The background level of this assay is <1000 RLU/mg protein and was defined by measuring luciferase activity in organs of non treated rats.

LacZ expression assay

Forty-eight hours after Ad5 Adapt LacZ administration rats were sacrificed and organs were removed and cut in 2 mm sections. Sections were fixed in 10% phosphate buffered formalin (pH 7.0) for 60 min at room temperature and incubated overnight in 0.5 M sucrose. The samples were subsequently frozen in liquid nitrogen. Ten μm thick frozen sections were prepared and stained with 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-gal) solution (Molecular Probes, The Netherlands) overnight at 37°C. Finally, sections were counterstained with Hematoxylin-Phloxin-Safran. The number of blue nuclei in each section was determined on digitalised photographs of four 20 × magnification fields with the interlobular vein in the centre (designated portal area) and of four fields centred on the central vein (called centro-lobular area). These two areas were slightly overlapping. The resolution of the photographs, which were processed in Adobe Photoshop 5.0, was 600 dots per inch (dpi). Cells were considered positive when a blue staining was seen in the nucleus. The total number of hepatocytes per field was determined by dividing the surface of the tissue section by the average surface of a hepatocyte. The surface of the lumen of large vessels was subtracted from the total surface of the tissue section. The average surface of the hepatocytes was determined by ten measurements of mononuclear hepatocytes per field. The surface measurements were performed with the NIH Image 1.62 software. It was shown in rats that binuclear hepatocytes have double the volume of mononuclear hepatocytes and that the extrahepatocytic space volume was 15% of the total liver volume [10].

To compare β-Galactosidase expression levels in the livers of Wag/Rij and Brown Norway rats, frozen livers were cut into sections with a thickness of 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 μm. Whole sections were scanned at 600 dpi under TIFF format and analysed in Adobe Photoshop 6.0. We quantified the blue staining intensity by performing the histogram of the luminosity and of the four colours on the whole liver section. Matching the histograms of a Brown Norway section to a Wag/Rij section with different thickness determine the relative liver β-Galactosidase intensity of the two strains.

Elisa assays

An mhATF-BPTI enzyme-linked immunoabsorbent assay (ELISA) was developed by Dr. P. Quax (Toegepast Natuurwetenschappelijk Onderzoek institute), using a monoclonal antibody specific for the ATF as the capture antibody and a polyclonal antibody directed against BPTI as the detector antibody [18]. As a standard we used medium of Ad5 Adapt mhAB infected CHO cell culture in which the mhATF-BPTI concentration was determined by an urokinase ELISA. Secretion of human endostatin in

plasma was routinely determined using a commercial ELISA kit (InvivoGen, CA, USA) according to the manufacturer procedure. INF-γ levels in rat sera were measured by an ELISA kit (BioSource Netherlands, B.V., The Netherlands).

Real Time PCR analysis

The amount of adenoviral genomes per cell was determined by a multiplex real-time polymerase chain reaction [22]. Real-time PCR is based on the 5'-3' nuclease activity of AmpliTaq Gold polymerase, which allows it to cleave fluorogenic probes resulting in fluorescence. The amount of fluorescence obtained during the PCR reaction is representative to the amount of amplified DNA.

Total DNA of transduced rat organs was extracted by using a DNeasy Tissue Kit (Qiagen). The kit efficiency was verified by measuring the recovery of plasmid DNA mixed from a liver lysate. We confirmed that 80% of the total DNA can be extracted from liver samples, as described by the manufacturer. To amplify the adenoviral DNA, specific primers (Ad5Clip-F: 5'CGACGGATGTGGCAAAAGT3' and Ad5Clip-R: 5'CCTAAAACCGCGCGAAAA3') were designed by using the Primer Express Software (Perkin-Elmer, Foster City, CA, USA). A fluorogenic probe (Ad5Clip-Pr: 5'-VIC-CACCGGCGCACACCAAAAACG-TAMRA-3') was also designed by the Primer Express Software. To determine the amount of cellular DNA present in the sample a second pair of primers and a FAM-probe specific for 18S rDNA [22] were used. The PCR reaction mixture consisted of 1 × buffer A (Perkin-Elmer), 3 mM $MgCl_2$, 200 μM dNTPs, 90 nM of each adenovirus primer, 100 nM of each 18S rDNA primer, 200 nM of each probe, 0.6 U AmpliTaq Gold polymerase (Perkin-Elmer) and 5 μl of total DNA sample. As a standard, to determine the amount of adenoviral genomes and cellular DNA, a plasmid containing approximately 5000 bp of the left part of the Ad5 genome (pAdapt) was mixed with cellular DNA extracted from A549 cells. The PCR reaction was initiated with a hot start at 95°C for 10 min and involved 45 cycles of 15 s at 95°C and 1 min at 60°C.

Northern Blotting

The animals were sacrificed 2 days after vector administration and the liver was immediately collected and snap frozen in liquid nitrogen. The liver samples were cut and homogenised in Trizol reagent (Gibco Life Technologies) using a blender. Total RNA was isolated from the liver tissue homogenate by using the method developed by Chomoczynski and Sacchi [23]. Briefly, chloroform was added and the aqueous phase containing the RNA was recovered. RNA was precipitated with isopropyl alcohol and the pellet was resuspended in RNase free water. The RNA and DNA content were measured by spectrophotometric analysis.

The quantity of RNA loaded was 20 μg as determined by spectrophotometry. The RNA loaded represents a similar proportion of luciferase mRNA in both strains as the total concentrations of RNA are similar in the Wag/Rij (6.40 ± 1.43 μg/gr) and in the Brown Norway rats (6.55 ± 1.91 μg/gr). The RNA was run on a 1% agarose gel and transferred onto Hybond N^+ membrane by overnight transfer. The RNA was then fixed by UV irradiation. The hybridisation was performed overnight with one stranded [^{32}P]α-dATF Luciferase probe (1800 bp). The membrane was exposed to a X-ray film (Kodak) for 24 h and afterwards the membrane was scanned in a phosphorimager for quantification. Then the membrane was stripped 15 min at 65°C and prehybridised with salmon sperm DNA. Then a second hybridisation procedure was performed with the [^{32}P]α-dATF β-actine probe.

Pathology

The scoring of the liver damage was performed in Wag/Rij and Brown Norway rats 2 and 7 days after the intravenous injection of adenoviral vectors. The liver pieces were fixed in formalin 10%, embedded in paraffin and sections of 10 μm were stained with haematoxylin and eosin. The total damage score is a compilation of scores of apoptosis, vacuolar changes, nuclear condensation, anisonucleosis, megalocytosis, mitosis, and inflammation (Table 4).

List of abbreviations

ALAT: Alanin Amino Trasferase

ATF: Amino Terminal Fragment

BPTI: Bovine Pancreatic Trypsine Inhibitor

γGT: Gamma Glutamyl Transferease

iu: Infectious units

IU: International Units

iv: Intra venous

LDH: Lactate DesHydrogenase

mhATF-BPTI: Murinised human ATF-BPTI

Authors' Contributions

PL and JA participated in the all aspects of the study, AL performed the real time PCRs, MH critically evaluated the results and DvB is the principal investigator.

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