Adenoviral gene therapy of the Tay–Sachs disease in hexosaminidase A-deficient knock-out mice

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The severe neurodegenerative disorder, Tays–Sachs disease, is caused by a β-hexosaminidase α-subunit deficiency which prevents the formation of lysosomal heterodimeric α–β enzyme, hexosaminidase A (HexA). No treatment is available for this fatal disease; however, gene therapy could represent a therapeutic approach. We previously have constructed and characterized, *in vitro*, adenoviral and retroviral vectors coding for α- and β-subunits of the human β-hexosaminidases. Here, we have determined the *in vivo* strategy which leads to the highest HexA activity in the maximum number of tissues in *hexA*-deficient knock-out mice. We demonstrated that intravenous co-administration of adenoviral vectors coding for both α- and β-subunits, resulting in preferential liver transduction, was essential to obtain the most successful results. Only the supply of both subunits allowed for HexA overexpression leading to massive secretion of the enzyme in serum, and full or partial enzymatic activity restoration in all peripheral tissues tested. The enzymatic correction was likely to be due to direct cellular transduction by adenoviral vectors and/or uptake of secreted HexA by different organs. These results confirmed that the liver was the preferential target organ to deliver a large amount of secreted proteins. In addition, the need to overexpress both subunits of heterodimeric proteins in order to obtain a high level of secretion in animals defective in only one subunit is emphasized. The endogenous non-defective subunit is otherwise limiting.

INTRODUCTION

Tay–Sachs disease is an inherited lysosomal storage disease characterized by deficiency of the ubiquitous lysosomal acid hydrolase, hexosaminidase A (HexA). The enzyme deficiency results primarily in the accumulation of one of its substrates, the G_{M2} ganglioside in neuronal cells (1), and leads to a diffuse apoptotic cell death phenomenon in the central nervous system (2). The disease is clinically variable, with severe forms showing profound mental retardation, and death within 2–3 years of birth (3).

HexA is one of the three isozymes of β-hexosaminidases. Each isozyme results from one of the different association possibilities of the α- and β-subunits. HexA, an α–β heterodimer, and hexosaminidase B (HexB), a β–β homodimer, are the two major forms of β-hexosaminidases. The α–α homodimer, hexosaminidase S, is a minor form which appears to have negligible catalytic activity (4). The α- and β-subunits are encoded by two distinct genes, *HEXA* and *HEXB*, located on different chromosomes (5).

Tay–Sachs disease is caused by mutations affecting the *HEXA* gene which lead to HexA deficiency. Like the majority of lysosomal storage disorder diseases, there is no treatment for this fatal disease. Several features of lysosomal diseases suggest that these pathologies are candidates for replacement or gene therapies. Firstly, a residual enzymatic activity of only 10% of the normal activity frequently is sufficient to avoid appearance of the clinical symptoms as exemplified by the so-called lysosomal enzyme pseudodeficiencies (6,7). Secondly, a part of each lysosomal enzyme is secreted and can be taken up by other cells via specific mannose-6-phosphate receptors (8) and probably by other means not yet identified (9,10). Therefore, ourselves and others (11) hypothesized that a pool of transduced cells overexpressing and secreting large amounts of enzyme would lead to a measurable activity in defective cells *in vivo*, via a secretion–recapture mechanism.

We previously have constructed adenoviral (12) and retroviral (13) vectors encoding the human α-subunit of β-hexosaminidases and demonstrated that these vectors were functional, allowing us to correct HexA deficiency in Tay–Sachs fibroblasts and to induce secretion of recombinant enzyme which was taken up by other deficient cells.

The murine model of Tay–Sachs disease obtained by knock-out of the *hexa* gene does not reproduce the severe symptoms of the human disease. This is due to the late accumulation of G_{M2} ganglioside at a low level in a few mouse brain structures (14–16). However, in this murine model, HexA activity is, as expected, null in all tissues. Therefore, in order to evaluate our strategy, we chose to administrate different combinations of adenoviral vectors via different routes to knock-out mice.

Here, we demonstrate that an efficient hexosaminidase secretion can be obtained by intravenous (i.v.), but not by intramuscular (i.m.), administration of the adenoviral vectors. Moreover, we show that co-transduction with the two vectors coding for the α-
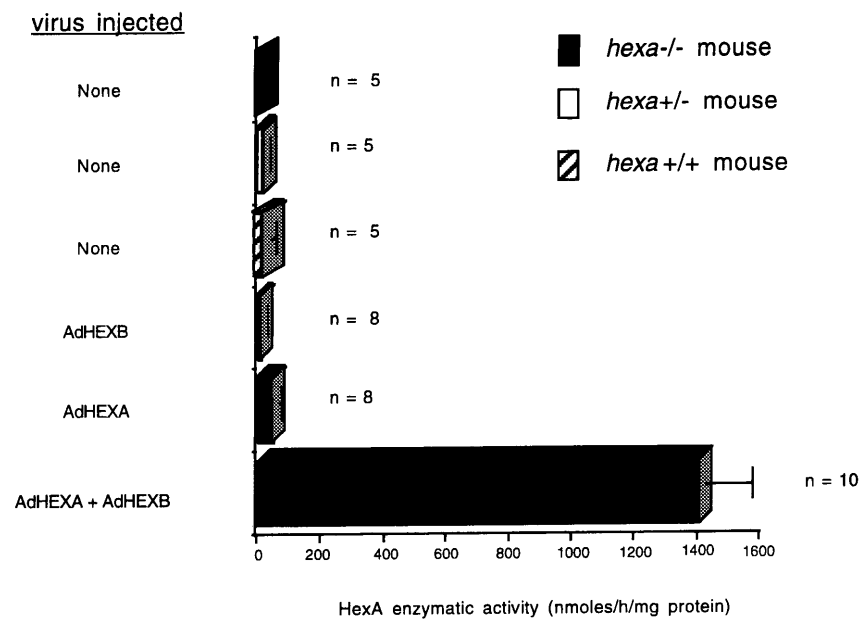
Figure 1. Histogram of hexosaminidase A activities in gastrocnemius muscle 8 weeks after i.m. injection of adenoviral vector in *hexa*^{−/−} mice. Enzymatic activities are measured with 4-MUGS as substrate, and values are means ± SD of the measurements in all animals of one group of mice. *n*, number of animals in each group.

and β-subunits, respectively, was required to obtain a high level of HexA synthesis and secretion. Upon transduction with the vector coding for the α-chain only, only limited amounts of functional enzyme were produced, suggesting that the β-chain was limiting. This approach allowed us to obtain complete HexA enzymatic correction through both direct transduction and secretion–recapture mechanisms. However, as already reported, neither adenoviral vectors (17,18) nor hexosaminidases (19) crossed the blood–brain barrier. Therefore, peripheral delivery of gene therapy vectors would be notably adapted to lysosome diseases without brain damage. Tay–Sachs and Sandhoff patients could nevertheless benefit since the efficacy of bone marrow transplantation in the Sandhoff mouse model suggests that correction of non-neuronal cells could improve health in spite of the brain lesions (20).

RESULTS

HexA enzyme activity in various tissues after i.v. injection of adenoviral vectors

HexA activity was measured with the 4-methyl-umbelliferyl-2-acetamido-2-deoxy-β-D-sulfoglucopyranoside (4-MUGS) substrate in the liver, spleen, heart, kidney, skeletal muscle (gastrocnemius) and brain after i.v. administration and is shown in Table 1. This activity was almost undetectable in all tissues tested from homozygous *hexa*^{−/−} mice, as compared with normal mice or heterozygous *hexa* knock-outs.
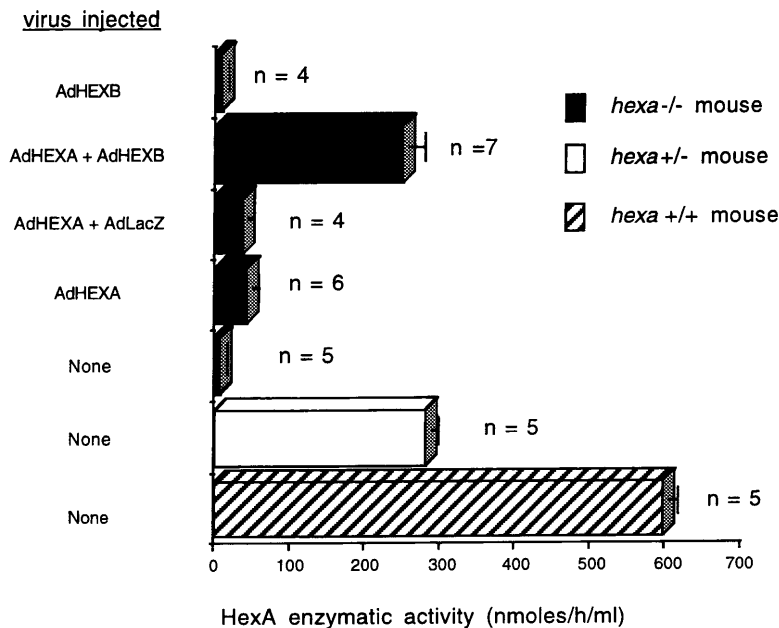
The *hexa*^{−/−} mice injected with adenoviral vectors were separated into four groups. Mice in the first group received 3×10^9 p.f.u. of AdHEXA (*n* = 6). HexA enzymatic activity was detected only in the liver (47% of the normal activity) and spleen (2% of the normal activity). In all other tissues, HexA activity was similar to that in non-injected *hexa*^{−/−} mice.

In the second group, *hexa*^{−/−} mice were given both 3×10^9 p.f.u. of AdHEXA and 1.5×10^9 p.f.u. of AdHEXB (*n* = 7). This co-injection resulted in a very high HexA activity in the liver (9-fold more than the normal value) and in partial or total correction in other tissues: 95% of the normal activity in the heart, 51% in skeletal muscle, 40% in spleen and 34% in kidney. In contrast, the activity in the brain was not significantly increased.

Table 1. Hexosaminidase A activities in tissues at 7 days after i.v. injection of adenoviral vectors in *hexa*^{−/−} mice

Animal	Liver	Spleen	Heart	Kidney	Muscle	Brain
hexa^{−/−}	0.7 ± 0.2	0.4 ± 0.2	0	0.6 ± 0.2	0.1 ± 0.0	2 ± 0.1
hexa^{+/−}	75.6 ± 13.6	271 ± 13.2	16.8 ± 0.5	34.3 ± 3.9	15.3 ± 2.7	31 ± 1.8
hexa^{+/+}	138 ± 11.3	515 ± 18	28.3 ± 1.1	63.5 ± 2.0	28 ± 1.6	65.6 ± 3.6
hexa^{−/−} (AdHEXA)	64.7 ± 8.9	16.6 ± 1.1	0.7 ± 0.2	1.3 ± 0.1	0.2 ± 0.0	2.2 ± 0.1
hexa^{−/−} (AdHEXA + AdLacZ)	59.7 ± 7.5	16.1 ± 1.2	0.9 ± 0.1	1.2 ± 0.1	0.2 ± 0.0	2.3 ± 0.2
hexa^{−/−} (AdHEXA + AdHEXB)	1235 ± 190	207 ± 21.7	26.8 ± 1.4	21.9 ± 1.6	14.4 ± 0.2	2.9 ± 0.4
hexa^{−/−} (AdHEXB)	5.4 ± 0.3	1.8 ± 0.3	0	1.1 ± 0.2	0.1 ± 0.0	2 ± 0.2

HexA activity was measured with the α-subunit-specific artificial fluorogenic substrate 4-MUGS. The enzymatic values are means ± SD of the measurements in all animals of one group of mice.

Figure 2. Histogram of hexosaminidase A activities in serum 7 days after i.v. injection of adenoviral vectors in $hexa^{-/-}$ mice. Enzymatic activities are measured with 4-MUGS as substrate, and values are means ± SD of values of the measurements in all animals of one group of mice. n, number of animals in each group.

In the third group, used as a first control group, mice received both 3×10^9 p.f.u. of AdHEXA and 1.5×10^9 p.f.u. of AdLacZ ($n = 4$). As expected, the results obtained were similar to those obtained for the first group of animals, demonstrating that the improvement observed upon co-injection of AdHEXA and AdHEXB was specific to the HexB transgene.

In the fourth group, an additional control, $hexa^{-/-}$ mice received 1.5×10^9 p.f.u. of AdHEXB. HexA levels in all tissues tested remained similar to those in non-injected $hexa^{-/-}$ tissues, indicating that synthesis of only the β-subunit did not increase the tissues' HexA activity, whereas it increased, as expected, the total liver hexosaminidase activity assayed with 4-methyl-umbellifer-yl-2-acetamido-2-deoxy-β-d-glucopyranoside (4-MUG) sub-strate: 501 ± 45 nmol/h/mg protein in AdHEXB-injected $hexa^{-/-}$ mice and 64 ± 6 nmol/h/mg protein in non-injected $hexa^{-/-}$ mice.

HexA activity in injected muscles

The $hexa^{-/-}$ mice received either 4.5×10^8 p.f.u. of AdHEXA ($n = 4$), 2×10^8 p.f.u. of AdHEXB ($n = 5$) or both by i.m. injection during the neonatal period (days 4–7). HexA activity assayed with the 4-MUGS substrate in the injected gastrocnemius muscle is shown in Figure 1.

Injection of AdHEXA corrected HexA activity, whilst co-injection of both AdHEXA and AdHEXB resulted in a strong overexpression. AdHexB alone slightly increased HexA activity, most likely due to a large accumulation of β-subunit in injected muscle and a weak affinity of HexB for the α-subunit-specific artificial substrate (4-MUGS) (21). No HexA activity was detected in livers isolated from animals injected i.m. (data not shown).

Secretion of hexosaminidase A in the bloodstream

Seven days after i.v. injection of adenoviral vectors, serum samples were analyzed for HexA activity; the results presented in Figure 2 were compared with HexA activity levels in wild-type and heterozygous mice. Non-injected $hexa^{-/-}$ mice showed a very low, probably aspecific enzyme activity. Double injection of $hexa^{-/-}$ mice with AdHEXA and AdHEXB vectors resulted in partial restoration of serum HexA activity, equivalent to 89% of the activity in heterozygous $hexA^{+/-}$ mice and 42% of that in wild-type mice. In contrast, serum HexA activity in $hexa^{-/-}$ mice injected either with AdHEXA or with AdHEXA and AdLacZ was only slightly increased, to 6–8% of the normal activity. AdHEXB i.v. injection alone has no effect on serum HexA activity. No HexA activity was detected in serum samples taken at 2 or 8 weeks after i.m. injection in $hexa^{-/-}$ mice, whatever the combination of adenoviral vectors (data not shown).

Different expression patterns of the human α-subunit in transduced liver and muscle

Western blot analysis of the human α-subunit in the liver and muscle was performed with a goat anti-human α-chain-specific antibody (Fig. 3). No signal was detected in samples from non-injected $hexa^{-/-}$ mice (lane 1) or AdHEXB-injected $hexa^{-/-}$ mice (lane 7). One 54 kDa band corresponding to the mature form of the human α-subunit of β-hexosaminidases was detected in samples from mice co-injected with AdHEXA and AdHEXB (lanes 4, 5 and 6). The intensity of this band appeared to be consistent with HexA activity: 2143, 1475 and 866 nmol/h/mg for samples in lanes 4, 5 and 6, respectively. The sensitivity of this western blot did not allow for detection of α-subunits in the normal liver of $hexa^{-/-}$ mice injected with AdHEXA with or without AdLacZ (lanes 2 and 3). Accordingly, HexA activity in
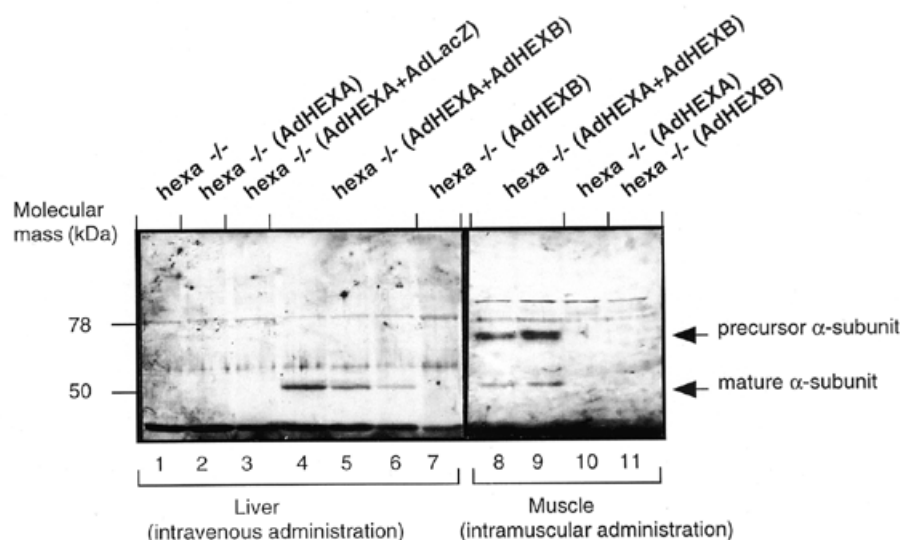
Figure 3. Immunodetection of human-β-hexosaminidase α-subunit in liver protein extracts after i.v. adenoviral vector injection or in gastrocnemius muscle after i.m. adenoviral vector injection in *hexa*$^{-/-}$ mice. Protein extracts (20 μg) were electrophoresed and β-hexosaminidase α-subunit immunodetection was performed with a goat anti-human α-subunit primary antibody used at 1:750 dilution, and an HRP-conjugated rabbit anti-goat IgG by ECL.

these conditions was 6- to 20-fold lower than that of the sample loaded in lane 6.

In the gastrocnemius muscle of i.m. injected mice, two different bands of 54 and 67 kDa were found at a ratio of 1:3.5, detected only after co-injection with AdHEXA and AdHEXB (lanes 8 and 9). These specific bands correspond to the mature and precursor forms of the α-subunit, respectively. The 67 kDa band is relatively fuzzy and may correspond to different precursor forms of quasi-equivalent molecular mass. These bands were not detected in muscle extracts from AdHEXA-injected mice, probably for the same reason as explained above.

Failure to detect the α-subunit in mice injected with AdHEXA alone suggests that human α-subunit is unstable *in vivo* when not associated with the β-subunit.

β-Hexosaminidase isozymic patterns in transduced tissues and in serum

To analyze the different forms of β-hexosaminidases synthesized by the liver after i.v. injection or by muscle after i.m. injection, hexosaminidase isoenzymes were separated by cellulose acetate electrophoresis (Fig. 4A). This figure shows human HexB (β–β) and HexA (α–β) from normal human fibroblasts (lane 1), and murine liver HexB (lane 2) from non-injected *hexa*$^{-/-}$ mice. Murine liver HexB co-migrates with human HexB. In the liver of AdHEXA-injected *hexa*$^{-/-}$ mice (lane 3); the faster band co-migrating with human HexA was the human α–murine β hybrid HexA.

Residual murine β–β (HexB) was at the limit of detection, most likely due to titration of the β-subunit by human interspecific HexA, as we have previously demonstrated (13). Similar results were observed in the liver of *hexa*$^{-/-}$ mice co-injected with AdHEXA and AdLacZ (data not shown). Much more intense HexA and HexB bands were detected after co-administration of AdHEXA and AdHEXB (lane 4). While electrophoretic migration does not allow for distinction between human and murine

HexB, and between human and hybrid HexA, the intensity of the bands indicates that most of the isoforms corresponded to the human enzymes. In *hexa*$^{-/-}$ mice injected with AdHEXB, a strong HexB band was detected (lane 5).

In the muscle, HexB was not detected in non-injected *hexa*$^{-/-}$ mice (lane 6), but both intense HexA and HexB isoforms appeared after i.m. co-injection of AdHexA and AdHexB (lane 7).

The same technique was used to analyze the different secreted β-hexosaminidase forms (Fig. 4B). The murine HexB precursor was the only isoform detected in the serum of non-injected *hexa*$^{-/-}$ mice (lane 5), co-migrating with the human HexB precursor (lane 1). Injection of AdHEXA did not result in secretion of any isoform other than the HexB precursor, whose secretion was increased after injection of AdHEXB (lanes 3 and 4, respectively). The serum of mice receiving both AdHEXA and AdHEXB (lane 2) revealed abundant isoforms identified as HexA and HexB precursors when compared with the electrophoretic pattern in normal human serum (lane 1).

DISCUSSION

Gene therapy of lysosomal diseases ideally would require transduction of most cells of the organism by vectors encoding the deficient enzyme, which remains to date an unrealistic goal. Another therapeutic prospect would consist of transduction of an important amount of cells in a large organ (liver, muscle), allowing them to produce and release the enzyme which is lacking into the bloodstream. This secreted enzyme could then be taken up by other tissues, and therefore correct the defect of remote non-transduced cells. In order to evaluate the efficacy of this latter approach, we have injected adenoviral vectors coding for hexosaminidase subunits into *hexa*$^{-/-}$ mice deficient for HexA (16).

First generation adenoviral vectors are known to cause marked cytotoxic and immune responses and provide only transient expression (22–25). In adult mice injected i.v., we therefore

A

B
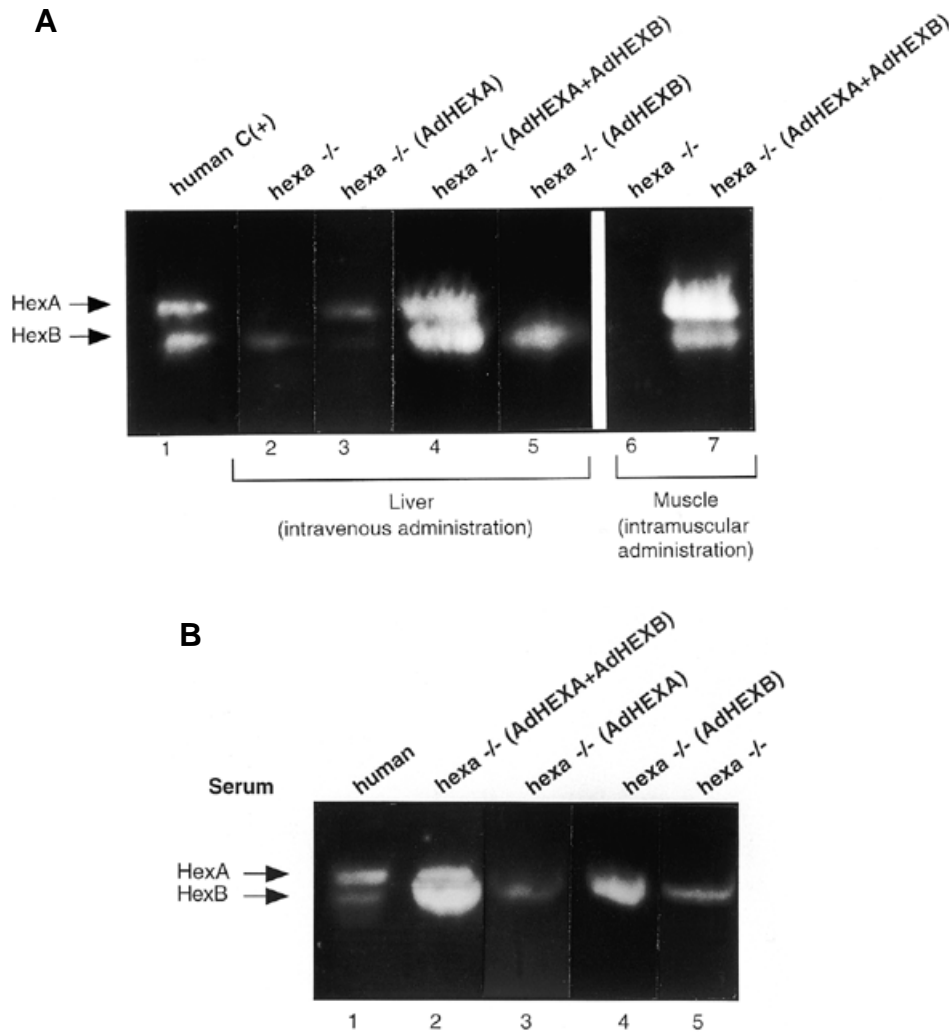
Figure 4. (**A**) Electrophoretic separation of β-hexosaminidase forms in liver protein extracts after i.v. adenoviral vector injection and or in gastrocnemius muscle after i.m. adenoviral vector injection in *hexa*$^{-/-}$ mice. (**B**) Electrophoretic analysis of serum hexosaminidases after i.v. adenoviral vector injection in *hexa*$^{-/-}$ mice. (**A**) Before loading on cellulose acetate gel, liver and muscle protein extracts of a *hexa*$^{-/-}$ mouse and liver protein extract of a *hexa*$^{-/-}$ AdEXA-injected mouse were concentrated 3-fold compared with other loaded protein extracts. On the left, the positions of human HexB and human HexA present in the non-deficient human fibroblasts are indicated (lane 1). Lane 2, liver protein extract from a non-injected *hexa*$^{-/-}$ mouse, with the presence of murine HexB which co-migrates with human HexB. Lane 3, liver protein extract from a *hexa*$^{-/-}$ mouse injected with AdHEXA. Two bands are detected; the more slowly migrating band is the murine HexB and the faster most likely corresponds to human–murine α–β interspecific HexA. Lane 4, liver protein extract from a *hexa*$^{-/-}$ mouse injected with both AdHEXA and AdHEXB. Two very intense bands can be detected: the more slowly migrating band corresponding to HexB (especially human but also murine and possibly human–murine β–β interspecific HexB) and the faster corresponding to HexA (primarily human but also probably interspecific HexA). Lane 5, liver protein extract from a *hexa*$^{-/-}$ mouse injected with AdHEXB, with the presence of one intense band corresponding to HexB (primarily human but also murine and possibly human–murine β–β interspecific HexB). Lane 6, muscle protein extract of a non-injected *hexa*$^{-/-}$ mouse. No band can be detected even after concentration of the extract, due to such a low amount of murine HexB in muscle and to the limited sensitivity of this technique. Lane 7, muscle protein extract of a *hexa*$^{-/-}$ mouse injected with both AdHEXA and AdHEXB. Two intense bands are detected corresponding to human HexB (more slowly migrating band) and HexA (faster migrating band). (**B**) Human HexB and HexA precursor forms are detected in normal human serum (lane 1) as the murine HexB precursor form is the only β-hexosaminidase form detected in non-injected *hexa*$^{-/-}$ mouse serum (lane 5). Lane 2, serum of a *hexa*$^{-/-}$ mouse injected with both AdHEXA and AdHEXB. Two bands are detected corresponding to the human HexA precursor form and both murine and human HexB precursor forms. Lane 3, serum of an AdHEXA-injected *hexa*$^{-/-}$ mouse. Only the murine HexB precursor form is detected due to the low sensitivity of the technique. Lane 4, serum of an AdHEXB-injected *hexa*$^{-/-}$ mouse, with the presence of an intense band corresponding to both the murine and human HexB precursor forms.

studied the level of enzyme correction in various organs and in serum at day 7 after injection.

Surprisingly, administration of AdHEXA alone by i.v. injection allowed for only a partial correction of HexA activity in the liver of *hexa*$^{-/-}$ mice (47%). HexA activity detected in other peripheral tissues and in the serum was very weak or null, respectively. This low level of liver HexA activity was not due to the inability of the human α-chain to associate with the murine β-subunit, since

synthesis of interspecific human–mouse HexA was detected by acetate electrophoresis. Interestingly, we also demonstrated that i.v. co-injection of AdHEXA and AdHEXB allowed for an overexpression of HexA in the liver, and for the correction of HexA enzymatic activity in all peripheral tissues tested, i.e. the liver, heart, spleen, skeletal muscle and kidney. It is noteworthy that the minimal rate of enzymatic correction in peripheral tissues (34%) could be largely higher than the theoretically needed

therapeutic threshold for Tay–Sachs disease and for other lysosomal storage diseases. Indeed, a 10% residual activity of the deficient lysosomal enzymes has been shown previously to prevent the development of such diseases (6,7).

The requirement for co-transduction of AdHEXA and Ad-HEXB for effective HexA production indicates that the α-subunit produced in excess cannot heterodimerize with endogenous β-subunit present at low, limiting levels. The level of *hexb* mRNA is indeed extremely low in the murine liver and muscles (26,27). Accordingly, we detected a very weak HexB (β–β) activity in these organs in *hexa*^{−/−} mice (data not shown). Finally, we also demonstrated that overexpression of the α-subunit in transduced HexA-deficient murine fibroblasts leads to a depletion of HexB, i.e. β-subunit (13). The importance of this result may extend beyond the scope of hexosaminidase deficiencies and be of significance for other diseases which result from deficiency in one subunit of heterodimeric or heteromultimeric proteins, such as clotting factor XIII (28), fibrinogen (29) or globin chains (30,31).

Partial restoration of HexA enzymatic activity was found in skeletal muscle and kidney after i.v. co-administration of AdHEXA and AdHEXB. This correction was most likely related to an enzyme uptake mechanism by these tissues from the bloodstream rather than to their direct transduction by the adenoviral particles. Intravenous injection of recombinant adenovirus is well known to target the liver rather than the kidney or muscle (17,18). Accordingly, 7 days after retro-orbital plexus injection of 3×10^9 p.f.u. of AdLacZ in four *hexa*^{−/−} mice, liver was transduced at 40%, whereas very few transduced muscle cells and no transduced kidney cells were detected (data not shown). Two further arguments can be raised to support the hypothesis of an uptake mechanism: (i) a high level of HexA enzymatic activity was detected in the serum of *hexa*^{−/−} mice submitted to i.v. co-administration of both AdHEXA and AdHEXB; and (ii) the uptake of HexA from the bloodstream has been demonstrated previously after i.v. injection of purified HexA in patients suffering from Sandhoff disease, characterized by HexA and HexB deficiency (19). This uptake process was also described *in vivo* for other lysosomal enzyme deficiencies treated by substitutive enzymatic therapy (32–34). In other tissues such as the liver, or in organs transduced less efficiently by adenoviral vectors, such as the spleen and the heart, it is likely that the detected HexA activity is accounted for by both viral transduction and enzyme uptake.

Adenoviral gene transfer into the muscle of newborn animals has been reported by several groups to allow for a better transduction than that for adult muscle (35) and systemic release of therapeutic proteins, such as factor IX, erythropoietin and neurotrophin 3 (36–39). We therefore investigated whether i.m. adenoviral administration of AdHEXA and AdHEXB in these optimal conditions could induce therapeutic synthesis of HexA and systemic secretion. Surprisingly, whereas injected muscles overproduced HexA, no enzymatic activity was detected in either serum or peripheral organs, such as the liver. In contrast, i.v. administration of both recombinant adenoviruses was associated with a highly significant release of HexA into the bloodstream. In hepatocytes, which are transduced preferentially after i.v. injection, mature human α-subunit is the only form detected. We did not detect the precursor form, probably because it is secreted rapidly, reflecting the high HexA systemic release by the liver. In contrast, human α-subunit accumulates in its precursor form in

transduced muscles, suggesting poor or no release into the bloodstream. In this case, muscle appears to be unsuitable for the production of HexA recombinant protein after gene transfer, most likely due to the size of the precursor forms (M_r of the HexA precursor is 120 ± 20 kDa) or to the complex glycosylation. The same observation recently has been demonstrated for one other lysosomal enzyme, α-glucosidase (40).

We report here for the first time the correction of HexA activity at the therapeutic level in peripheral tissues after adenoviral gene transfer. This correction, however, has not been evaluated in the long term because of the known non-persistence of transgene expression after first generation adenoviral vector transfer. In these experiments, HexA deficiency was not corrected in the brain. This is consistent with the absence of brain transduction following i.v. adenoviral administration (17,18), and with the absence of blood–brain barrier transport for HexA that has been described previously (19). However, a recent study has suggested that neuronal G_{M2} ganglioside accumulation is not responsible for all G_{M2} gangliosidosis symptoms (20). In that study, Proia *et al*. (20) raised the hypothesis that Tay–Sachs disease could be due to the presence of a toxic HexA substrate, such as lyso-G_{M2} ganglioside, which could be synthesized and secreted by peripheral tissues. Should this hypothesis be true, peripheral enzymatic HexA correction at the therapeutic level could induce the degradation of such a toxic compound and thereby inhibit its accumulation. This hypothesis, however, cannot be tested in *hexa*^{−/−} mice, which show no neurological phenotype. On the other hand, if HexA delivery to the central nervous system remains a necessity, it could be achieved by obtaining direct synthesis of HexA by neuronal cells, i.e. by injection of progenitor neuronal cells into the brain (41,42), or by the synthesis in peripheral tissues of a modified HexA protein capable of reaching the central nervous system through the blood–brain barrier (43–45).

In conclusion, we show that it is possible to obtain the restoration of a theoretically therapeutic enzymatic activity in several peripheral tissues in the mouse model of HexA deficiency. Our study demonstrates that this restoration requires the administration of both α- and β-subunits of β-hexosaminidases, and suggests that the liver could be an effective organ for overexpression and secretion of a protein into the bloodstream which could then be taken up by peripheral tissues. We currently are testing this approach on the HexA/HexB-deficient mouse model obtained by disruption of the *hexb* murine gene (15,46). These animals reproduce the Tay–Sachs phenotype more faithfully than the α-subunit-deficient mice (47). In addition, they display both peripheral and central disorders such that the efficacy of peripheral delivery of the subunits which are lacking in both types of pathology will be easy to check.

MATERIALS AND METHODS

Adenoviral vectors

The three recombinant adenoviral vectors, AdHEXA, AdHEXB and AdLacZ, are E1/E3 deleted. The adenoviral vector AdHEXA coding for the human α-subunit of β-hexosaminidases has been described previously (12). Briefly, the *HEXA* coding sequence was inserted into the E1 region and its expression was driven by the RSV long terminal repeat promoter. For the plasmid pAdCMV, HEXB was constructed by E. Kremer and C. Drugan

and will be presented in detail elsewhere. In this vector, the *HEXB* cDNA was under the control of the cytomegalovirus promoter (CMV). The AdLacZ (22), used as a control vector, encoded *Escherichia coli* β-galactosidase targeted to the nucleus under the control of the CMV promoter.

Mice

Homozygous *hexa* knock-out mice (*hexa*$^{-/-}$) have been described previously (16). *hexa*$^{+/-}$ mice were bred in a 129/(C57BL/6) background. Double mutants (*hexa*$^{-/-}$) were identified by PCR analysis on tail DNA (16). Wild-type and heterozygous (*hexa*$^{+/-}$) littermates were used as controls in the analyses.

Adenoviral vector injections

For i.v. injections, 8-week-old *hexa*$^{-/-}$ mice were injected with a total volume of 200 µl in the retro-orbital plexus. Mice received injection of 3×10^9 p.f.u. of AdHEXA, 1.5×10^9 p.f.u. of AdHEXB or 3×10^9 p.f.u. of AdLacZ, or co-injection of either 3×10^9 p.f.u. of AdHEXA + 1.5×10^9 p.f.u. of AdHEXB, or 3×10^9 p.f.u. of AdHEXA + 1.5×10^9 p.f.u. of AdLacZ. Seven days later, mice were killed and tissues were isolated after animal infusion with physiological serum (0.9% NaCl, pH 7.4).

For i.m. injections (37), mice of age 3–5 days were used. Adenoviral vectors were injected into both gastrocnemius muscles of each mouse briefly anaesthetized by hypothermia. Mice received injection of 4.5×10^8 p.f.u. of AdHEXA or 2×10^8 p.f.u. of AdHEXB, or co-injection of 4.5×10^8 p.f.u. of AdHEXA + 2×10^8 p.f.u. of AdHEXB in a volume of 50 µl in each gastrocnemius muscle. Eight weeks later, mice were killed and organs were removed.

Animal care and experiments were performed in accordance with the French Ministry of Agriculture.

Biochemical analysis of hexosaminidase activities

Isolated tissues were homogenized in 10^{-2} M phosphate buffer, pH 6, in the presence of 1% (v/v) NP-40. The homogenates were spun at 21 000 *g* for 1 h at 4°C. The clear supernatants represent the protein extracts used for all protein experiments. Protein concentration was determined by Coomassie protein assay kit (Pierce, Rockford, IL).

Blood samples collected from the retro-orbital plexus were spun twice at 900 *g* for 15 min at 4°C in order to obtain the serum samples (supernatant). Ten microlitres of protein extract diluted at 4 mg/ml and 10 µl of serum were assayed fluorometrically for enzymatic activities. Artificial substrates were used, 4-MUG to determine β-hexosaminidase activities, or 4-MUGS, the α-sub-unit-specific substrate, to determine HexA activity, as previously described (48). Specific enzymatic activities were calculated as nmol/h/mg protein from protein extracts and as nmol/h/ml from serum samples.

Western blot analysis

Twenty micrograms of protein extracts was denatured and fractionated by SDS–PAGE on a 9% (w/v) gel. After electrophoresis, proteins were transferred to Hybond C filters (Amersham Life Science, Buckinghamshire, UK) and incubated with a purified goat anti-human β-hexosaminidase α-subunit antibody (49). The specific immune complexes were detected using a rabbit anti-goat IgG horseradish peroxidase-conjugated secondary antibody by enhanced chemiluminescence.

Electrophoresis of β-hexosaminidases

Protein extracts were concentrated or not three times by centrifugation on 50 kDa millicon columns in order to load the same protein extract volumes (20 or 60 µg) on cellulose acetate strips (Cellogel; Chemetron, Milan, Italy). Electrophoresis was performed as previously described (50) for 2 h at 4°C under 200 V in 0.04 M phosphate buffer, pH 6, followed by incubation with 4-MUG substrate.

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