Supplementary Data

High-capacity adenoviral vector

Details of the molecular characterization, rescue, and amplification of the high-capacity, helper-dependent adenoviral (HC-Ad) vector were published previously (Xiong et al., 2006; Palmer and Ng, 2008, 2011; Southgate et al., 2008). The vectors used in the phase I clinical trial will be produced using a system identical to that used to produce the vectors used in the present study. The major differences between the two batches of vectors will be that the vectors prepared specifically for the clinical trial will be subjected to more thorough testing for contamination and also that they will be produced under Good Manufacturing Practice conditions at the Baylor College of Medicine Vector Core facility. HC-Ad-TetOn-Flt3L expresses the human soluble fms-like tyrosine kinase 3 ligand (Flt3L) under the control of the tightly regulatable mCMV-TetOn inducible expression system (Xiong et al., 2006).

Animals

Adult male Lewis rats (200–300 g; Harlan) were used. Rats were kept in controlled conditions of light (12-hr light–dark cycles) and temperature (20–25°C). Rats received water and standard rodent chow ad libitum. All animal procedures were carried out in accordance with National Institutes of Health guidelines for the care and use of laboratory animals and approved by the University of Michigan Unit for Laboratory Animal Medicine.

Feeding trial in Lewis rats

Starting on day 0, all rat chow was removed from each cage and replaced with 500 g doxycycline (DOX) chow (TestDiet). On the following day, the chow remaining in the cage was weighed and recorded, and the weight of each rat was also recorded. The daily weighing of the chow and rats continued for 2 weeks. The average amount of chow consumed per rat was calculated by dividing the chow intake per cage by the number of rats in that cage. The average DOX intake from the chow was calculated by multiplying the daily average chow intake by the conversion factor 1,000 ppm (1 mg DOX/1 g chow).

Tissue harvesting

Animals were perfused/fixed under anesthesia with ketamine (75 mg/kg IP) and dexmedetomidine (0.25 mg/kg IP). Animals were perfused with oxygenated Tyrode's solution (132 nM NaCl, 1.8 mM CaCl$_2$, 0.32 mM NaH$_2$PO$_4$, 5.56 mM glucose, 11.6 mM NaHCO$_3$, 2.68 mM KCl, 0.1 U/ml heparin, pH 7.4) followed by fixation with 4% paraformaldehyde (4% paraformaldehyde, 137 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer, pH 7.4). Brains were postfixed in 4% paraformaldehyde for 3 days before processing.

Neuropathological analysis

Fifty-micrometer serial coronal sections were cut at the immediate vicinity of the injection site and free-floating immunocytochemistry was performed as previously described (Candolfi et al., 2006; King et al., 2008a, 2008b; Puntel et al., 2010a) with markers for oligodendrocytes and myelin sheaths (mouse monoclonal anti-MBP, 1:1,000; Chemicon; Cat. # MAB1580), dopaminergic nerve terminals (rabbit polyclonal anti-TH, 1:5,000; Calbiochem; Cat. # 657012), CD8$^+$ T cells (mouse anti-CD8, 1:1,000; Serotec; Cat. # MCA48G), macrophages and microglia (CD68/ED1, mouse anti-ED1, 1:1,000; Serotec; Cat. # MCA341R; IBA1, polyclonal rabbit anti-IBA1, 1:1,000; Wako Pure Chemical Industries; Cat. # 019-19741), activated macrophages, microglia, and immune cells (mouse anti-MHC II, 1:1,000; Serotec; Cat. # MCA46G), and Flt3L [rabbit anti-Flt3L, 1:500, custom made (Curtin et al., 2006)]. NISSL staining was performed to assess gross histopathological features of each brain (Ghulam Muhammad et al., 2009). The stained sections were photographed with a Carl Zeiss Optical Axioplan microscope using Axiovision Rel 4.6 and MOSAIX software (Carl Zeiss).

Expression of Flt3L in the striatum

Allometric rat doses of DOX were calculated by dividing the human DOX dose by the average human weight of 45.45 kg and then multiplying by the allometric conversion factor (Voisin et al., 1990) of 7. Therefore, a dose of 300 mg of DOX in humans is equivalent to 300 mg/45.45 kg×7=46.2 mg/kg in the rat. Similarly, a 200 mg dose of DOX in humans is equivalent to 30.8 mg/kg in the rat. We prepared suspensions of the DOX solution (DOX hyclate; Sigma-Aldrich; Cat. # D9891) and administered the appropriate dose via oral gavage, three rats per DOX-dose group, 2 days before HC-Ad vector delivery (day −2). On day 0, rats were intracranially injected with 1×10^9 viral particles (vp) of HC-Ad-TetOn-Flt3L into the striatum. The vector was injected in a final volume of 1.5 μl of saline using a 5 μl Hamilton syringe fitted with a 26-gauge needle. The stereotactic coordinates were 1.0 mm anterior and 3.0 mm lateral to the bregma; the injection volume of 1.5 μl was delivered in three locations (0.5 μl each) at −5.5, −5.0, and −4.5 mm from the dura. The appropriate dose of DOX (water for the control animals) was administered to each animal every 12 hr until day 14, whereupon blood was drawn via the right atrium to determine the DOX levels in the plasma and perform serum chemistry analyses. Animals were then perfused/fixed as described above.

Serum biochemistry

Collected blood was transferred to serum separation tubes (SC Micro Tube Ser-Gel PP; Biotang; Cat. # 41.1378.005); samples were left for 30 min at room temperature to allow for blood coagulation before centrifugation at 2,000 Relative Centrifugal Force for 10 min and immediate analysis on a VetTest 8008 serum chemistry analyzer (IDEXX Laboratories).

Measurement of DOX concentration in plasma or DOX chow using liquid chromatography with tandem mass spectrometry

A 20 μl aliquot of each plasma sample or homogenized DOX chow sample was mixed with 20 μl of methanol before

adding 60 μl of acetonitrile-containing tetracycline as an internal standard. The samples were prepared in duplicate. To collect the supernatant for liquid chromatography and dual-mass spectrometry (LC-MS/MS) analysis, the suspensions were centrifuged at 15,000 rpm for 10 min. To ensure retention of DOX and its internal standard during LC-MS/MS, the final supernatant was mixed with water at 1:6 (v/v) ratio. LC-MS/MS analysis was performed using a 5 cm × 2.1 mm, 5 μm Zorbax C18 column (Agilent), a mobile phase A of 0.1% formic acid in purified water, a mobile phase B of 0.1% formic acid in acetonitrile, and a flow rate of 0.4 ml/min. This procedure was performed by the Pharmacokinetics Core at the University of Michigan.

Circulating neutralizing antiadenovirus antibodies

The level of adenovirus-specific neutralizing antibodies was assessed as described previously (Muhammad *et al.*, 2010; Puntel *et al.*, 2010a, 2010b). Briefly, plasma samples were heat-inactivated at 56°C for 30 min and serially diluted twofold in minimal essential medium (Invitrogen) containing 10% heat-inactivated fetal bovine serum. The range of dilutions was 1:1 to 1:4,096. Each 50 μl plasma dilution was incubated with 3×10^6 infectious units of first-generation Ads expressing β-galactosidase (Ad-β-Gal, 200 infectious units per cell) for 90 min at 37°C. A 50 μl aliquot of the plasma/Ad-β-Gal mixed sample was then added to the wells of a 96-well plate containing 1.5×10^4 preseeded HEK 293 cells per well and incubated at 37°C for 1 hr. A further 50 μl of medium containing 10% fetal bovine serum was added to each well, and the cells were incubated at 37°C for 20 hr before fixing with 1% glutaraldehyde in phosphate-buffered saline (pH 7.4) and staining with 5-bromo-4-chloro-indolyl-β-d-galactoside (X-gal; Sigma). The neutralizing antibody titer for each animal is given as the reciprocal of the highest dilution of serum at which 50% of maximal Ad-β-Gal-mediated transduction was inhibited. Serum from a preimmunized animal was used as a positive control, and serum from a naïve animal was used as a negative control. Serum titers of 64 and below were considered negative, and titers of 128 and above were considered positive (Nwanegbo *et al.*, 2004; Puntel *et al.*, 2013).

DOX concentration (ng/ml) in rat plasma is shown

Low (30.80 mg/kg/day) and high (46.20 mg/kg/day) doses of DOX were administered to naïve Lewis rats for 2 days before an intracranial injection with 1×10^9 vp of HC-Ad-TetOn-Flt3L. Both concentrations of DOX were administered for 14 days after surgery. Blood was collected 14 days postsurgery upon euthanasia and analyzed for DOX content by the Pharmacokinetics Core at the University of Michigan.

Good Laboratory Practice guidelines

This study was not performed under Good Laboratory Practice guidelines.

Statistical analysis

Overall differences in circulating anti-adenovirus-neutralizing antibody titers were assessed using the nonparametric Kruskall–Wallis test. Significance was assessed using a *p*-value of 0.05.

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