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Death switch for gene therapy: application to erythropoietin transgene expression

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Abstract

The effectiveness of the caspase-9-based artificial "death switch" as a safety measure for gene therapy based on the erythropoietin (Epo) hormone was tested *in vitro* and *in vivo* using the chemical inducer of dimerization, AP20187. Plasmids encoding the dimeric murine Epo, the tetracycline-controlled transactivator and inducible caspase 9 (ptet-mEpoD, ptet-tTAk and pSH1/Sn-E-Fv'-Fvls-casp9-E, respectively) were used in this study. AP20187 induced apoptosis of iCasp9-modified C2C12 myoblasts. *In vivo*, two groups of male C57Bl/6 mice, 8-12 weeks old, were injected intramuscularly with 5 µg/50 g ptet-mEpoD and 0.5 µg/50 g ptet-tTAk. There were 20 animals in group 1 and 36 animals in group 2. Animals from group 2 were also injected with the 6 µg/50 g iCasp9 plasmid. Seventy percent of the animals showed an increase in hematocrit of more than 65% for more than 15 weeks. AP20187 administration significantly reduced hematocrit and plasma Epo levels in 30% of the animals belonging to group 2. TUNEL-positive cells were detected in the muscle of at least 50% of the animals treated with AP20187. Doxycycline administration was efficient in controlling Epo secretion in both groups. We conclude that inducible caspase 9 did not interfere with gene transfer, gene expression or tetracycline control and may be used as a safety mechanism for gene therapy. However, more studies are necessary to improve the efficacy of this technique, for example, the use of lentivirus vector.

Key words: Gene therapy; Erythropoietin; Death switch; Caspase 9; AP20187; Anemia

Introduction

The eventual adoption of gene therapy as a routine medical procedure requires the development of safe vectors and fail-safe mechanisms to eliminate potential injuries to the host, even by removing genetically altered cells. The ideal suicide switch should be non-immunogenic and non-toxic when not induced and able to trigger cell death, independently of tissue type or cell cycle stage. Chemically induced dimerization (CID) (1) is a versatile approach to the control of gene activity. A panel of inducible suicide genes based on Fas and Fas-signaling intermediates such as Apaf-1 and caspases 1, 3, 8, and 9 have been successfully developed (2-7). In our studies, an inducible caspase 9 (iCasp9) was the most CID-sensitive one (3, and Spencer DM, unpublished results). The iCasp9 consists of two mutated FK506-binding protein (FKBP)-12 domains, both containing an F36V mutation, fused to the procaspase 9 cDNA under the control of the broadly constitutive Sralpha promoter. The iCasp9 can be dimerized with AP1903 or AP20187, which are nontoxic, cell-permeable and synthetic dimeric FK506 analogues that have been designed to reduce interactions with endogenous FKBPs, while enhancing binding to the mutated FKBP variant (8). Administration of the dimerizing drug results in the aggregation of iCasp9 molecules, leading to their activation. Caspase 9 will subsequently activate downstream effector caspases such as caspase 3, and ultimately induce apoptosis (1,3,4,9). The drug AP1903, an analogue of AP20187, has been tested in 28 normal volunteers and has been shown to be safe and well tolerated (10).

The cost and quality of therapeutic protein production are major obstacles for the treatment of a large number of patients with a wide range of diseases. In this context, gene therapy for treating patients with hemophilia using factor IX or factor VIII has been increasingly improved, gaining space and importance in this field (11-13). Thus, millions of patients with chronic anemia caused by kidney diseases, cancer,

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AIDS, myelodysplasia, or even hemoglobinopathies (14-18) could benefit from the delivery of erythropoietin (Epo) by gene therapy, particularly in diseases where resistance to Epo is important and administration of large doses of this hormone is necessary in order to obtain a response.

In view of its wide application, gene therapy using the Epo gene has been the aim of many *in vitro* and *in vivo* studies (19-28). Sufficient gene expression for a long-term response has been easily obtained in most *in vivo* studies and the target of erythropoietin treatment is reached when the hormone is delivered to the plasma by intramuscular injection of the vector or implant of encapsulated transformed cells (19-28). The success of this approach has been demonstrated directly by hematocrit increase as well as by measurement of Epo protein in cell culture or in the serum of tested animals, such as mice, monkeys, and cats. However, the main side effects observed using nonviral or viral vectors for delivery of the gene are polycythemia and fatal thrombosis (19,20). Thus, the great challenge with this type of gene therapy is the achievement of down-regulation of Epo gene expression or the elimination of modified cells in cases of hemodynamic and rheologic problems associated with increased risk of thrombosis.

Therefore, the effectiveness of the caspase-9-based artificial "death switch", as a safety measure for gene therapy based on the erythropoietin hormone, was tested *in vitro* and *in vivo* using the chemical inducer of dimerization, AP20187.

Material and Methods

Plasmids

The following plasmids were used in this study: the murine dimeric Epo-encoding plasmid, ptet-mEpoD (25) (5 µg plasmid per 50 g weight), the tetracycline-controlled transactivator encoding, ptet-tTAk plasmid (0.5 µg/50 g; Gibco, France) (29), and pSH1/Sn-E-Fv'-Fvls-casp9-E, the iCasp9-encoding plasmid iCasp9 that consists of full-length iCasp9 including its caspase recruitment domain (CARD; GenBank NM001 229) linked to two molecules of a 12-kDa human FK506-binding protein (FKBP12; GenBank AH002 818) that contains an F36V mutation. For the *in vivo* studies, varying amounts of the iCasp9-encoding plasmid were used. The ptet-mEpoD plasmid was obtained by subcloning two PCR products of the complete murine Epo open-reading frame cDNA sequence, except for the stop codon, and except for the signal sequence, respectively, into the BS-KSII+ phagemid (Stratagene, Saint-Quentin, France), linked by a linker fragment encoding a peptide of 9 residues (Gly-Ser-Gly4-Ser-Gly-Ala). The final construct was obtained by subcloning the *Cla*I-*Hin*dIII mEpoD fragment from pBS-mEpoD in the ptet-splice plasmid (Gibco, France). The amounts of ptet-mEpoD and ptet-tTAk plasmids injected followed a previously described protocol (25). All plasmids were doubly purified using a cesium chloride gradient. The ptet-mEpoD and iCasp9 plasmids are represented in Figure 1.

C_2C_{12} cell lineage

Murine C_2C_{12} myoblasts derived from the skeletal leg muscle of an adult C3H mouse were obtained from the American Type Culture collection (ATCC, CRL 1777, USA) and grown in Dulbecco's modified Eagle's minimal essential medium with 10% fetal bovine serum at 37°C in a CO_2 incubator.

Stable transfections

Cells (5 x 10^5) were transfected with 20 µg iCasp9 and 1 µg tpMC1neoPolyA (Stratagene, France) vector in 1440 µL OptiMEM (Invitrogen Life Technologies, USA) and 60 µL lipofectamine (Invitrogen Life Technologies), according to manufacturer instructions. Genetically modified cells were selected with neomycin (Invitrogen Life Technologies) for 2-3 weeks and the clones were then expanded and tested by PCR and Western blot.

Animals

Eighty-nine male C57Bl/6 mice, 8-12 weeks old, were used in this study. All experiments were performed in agreement with the Ethics Committee of the Biology Institute of the University of Campinas.

Plasmid injections and electric-pulse delivery

Anesthesia and muscle plasmid delivery were performed as described in Payen et al. (24). The animals were anesthetized by an intraperitoneal injection of a mixture of ketamine (85.8 mg/kg) and xylazine (3.1 mg/kg) diluted in 150 mM NaCl. The vectors were injected into the tibial muscle of the animal in a final volume of 30 µL 150 mM NaCl using a Hamilton syringe. Electric pulses were delivered 30 s after injection using external plate electrodes placed on each side of the leg. As previously described, eight 20-ms and 200-V/cm electric pulses were applied at 1 Hz frequency using an ECM 830 electroporator (BTX Molecular Delivery Systems, USA).

Detection of the iCasp9 plasmid by PCR analysis

Genomic DNA obtained from stably transfected cells or animal muscles was subjected to PCR analysis using a pair of oligonucleotides designed in different exons, in order to give a fragment of 470 bp, specific for the detection of the iCasp9 plasmid. Sense (5' CGG AAA CAC CCA GAC CAG TG 3') and antisense (5' CGA CAC AGG GCA TCC ATC TG 3') primers were synthesized by Integrated DNA Tech (Coralville, USA) and the following PCR conditions were used: 35 cycles of 1-min denaturation at 94°C, 1-min annealing at 56°C and 1-min extension at 72°C, followed by a 5-min extension at 72°C. PCR products were run on 1.5% agarose gels and analyzed by staining with ethidium bromide and visualized using Kodak Digital Image 1D 3.0.2.

Detection of iCasp9 expression by Western blot analysis

For protein extraction, cells were incubated for 30 min at 4°C in lysis buffer containing 100 mM EDTA, 1% Triton in PBS, 0.5 M PMSF, 10 µg/mL aprotinin, 1 µg/mL leupeptin and centrifuged at 12,000 rpm for 20 min at 4°C. The supernatant was collected and frozen at -80°C. Equal amounts of protein were submitted to SDS-PAGE in a Laemmli system using 12% polyacrylamide. Western blots were carried out with a rabbit anti-caspase 9 polyclonal antibody (H-83, Santa Cruz Biotechnology, USA, 1/1000) and bands were developed with the ECL™ Western Blotting Analysis System (Amersham-Pharmacia Biotech, UK).

CID

The inducer of dimerization used in the present study was AP20187 (kindly provided by ARIAD Pharmaceuticals, USA). The AP20187 (Ariad Pharmaceuticals Kit Argent™) stock solution was prepared by dissolving the compound in 100% ethanol, at a concentration of 62.5 mg/mL and stored at -20°C. AP20187 was administered to the cell culture at 100 nM final concentration. For *in vivo* studies, the AP20187 stock solution was diluted in 2% Tween and PEG-400 to a final concentration of 0.25 mg/mL, and administered once intraperitoneally at the dose of 3 mg/kg. AP20187 was administered according to manufacturer instructions.

Doxycycline administration

In order to test the efficiency of the control of Epo secretion by doxycycline, despite co-adjuvant administration of iCasp9 and AP20187, 200 µg/mL doxycycline (Sigma, USA) was administrated in drinking water for 2 weeks to 2 animals that received 5 µg/50 g ptet-mEpoD plasmid and 0.5 µg/50 g ptet-tTAk plasmid and to 6 animals also injected with 6 µg/50 g iCasp9 plasmid.

Hematocrit determination

Hematocrit was determined weekly by a standard microhematocrit method.

Plasma Epo measurement

Plasma Epo concentration was determined using an ELISA kit and mouse Epo standards both from R&D Systems (Oxon, UK).

TUNEL assay

For *in vivo* studies, paraffin sections were dewaxed, treated with proteinase K and then nick-end labeled using fluorescein-conjugated 12-dUTP and the enzyme terminal deoxynucleotidyl transferase (TdT) (Promega, USA). Sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) to locate nuclei. Nuclei containing fragmented DNA present bright white fluorescence with

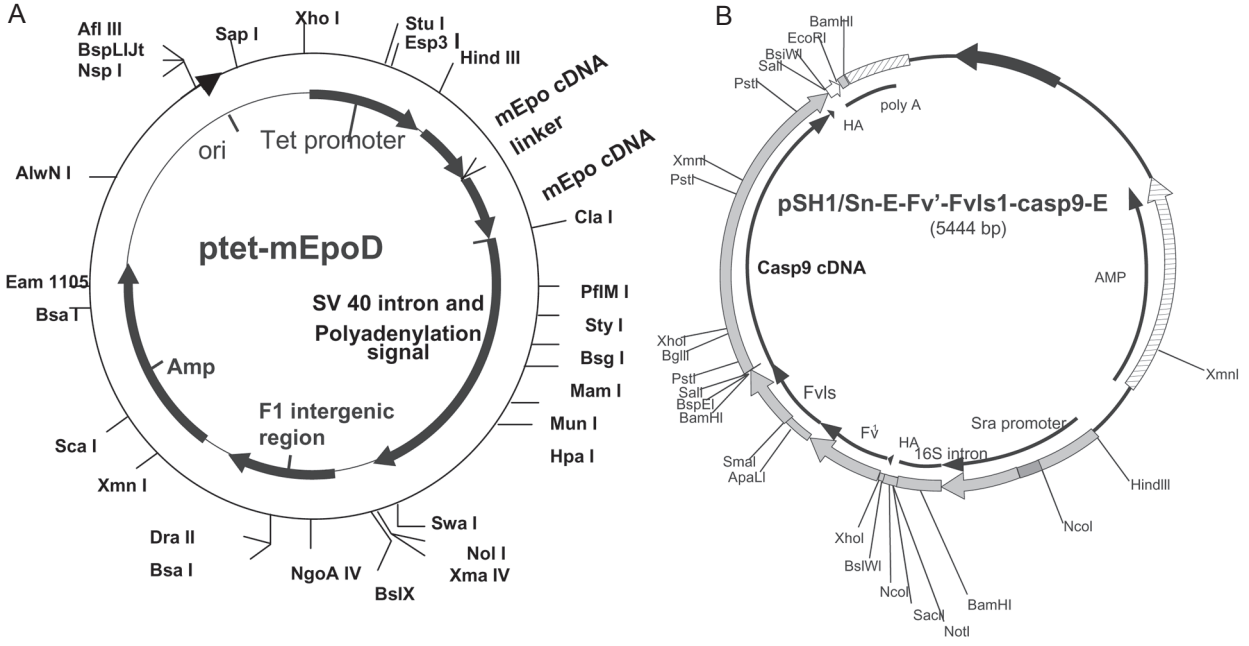

Figure 1. Structure of the ptet-mEpoD and iCasp9 plasmids used in the present study. *A*, The ptet-mEpoD plasmid contains two molecules of the complete murine erythropoietin (Epo) open-reading frame cDNA sequence linked by the peptide linker fragment encoding 9 amino acid residues (Gly-Ser-Gly4-Ser-Gly-Ala), subcloned in the ptet-splice plasmid (Gibco, Cergy-Pontoise, France). *B*, The iCasp9 plasmid consists of the full-length inducible caspase 9 including its caspase recruitment domain linked to two molecules of a 12-kDa human FK506-binding protein that contains an F36V mutation, under the control of the SR alpha promoter.

contribution from both green (fluorescein) and blue (DAPI) staining. For the *in vitro* study, 5 x 10^4 stably transfected cells were treated with 100 nM AP20187. After 12 h, the TUNEL reaction was performed according to the same protocol, with the exception that nuclei were counterstained with propidium iodide, so that apoptotic nuclei appeared yellowish-orange, with contribution from both green (fluorescein) and red propidium iodide. A positive reaction was attained by treating a tissue section with DNase I. For the negative control, the TdT enzyme was omitted from the incubation step. Images were obtained using an Olympus fluorescence microscope.

Statistical analysis

Data are reported as medians. For each group of mice, variables were analyzed by the Wilcoxon-signed rank test and results were considered significant when $P < 0.05$.

Results

In vitro studies

Stable transfections. Since the *in vivo* studies were designed to test the intramuscularly injected iCasp9 system, stable transfections in mouse myoblasts were performed. Thirty-nine clones of C_2C_{12} myoblasts transfected with iCasp9 were selected with the antibiotic neomycin. Using the PCR technique it was possible to detect the presence of the integrated transgene in 27 of these clones (Figure 2A). Western blotting using the anti-caspase 9 antibody for the detection of human caspase 9 revealed a chimeric protein with a molecular weight of ~70 kD, corresponding to 46 kD of the human caspase 9 and 12 kDa of each molecule of FKBP12, in 6 clones. Clones 1 and 3 (Figure 2B) were selected for CID treatment using 100 nM AP20187. The TUNEL assay was performed 12 h after treatment and most cells analyzed were positive (clone 1 treated - 88%, untreated - 3%; clone 3 treated - 78%, untreated - 5%). Figure 2C shows a representative field of clone 1, treated and untreated. These results demonstrate that the iCasp9 plasmid may be expressed in mouse myoblasts and presents a good response to CID.

In vivo studies

The design of Experiments 1 and 2 is represented in Figure 3.

Experiment 1

Definition of quantity of iCasp9 vector administration. In order to test the amount of iCasp9 to be injected in the animals without compromising the expression of the Epo vector, 3 groups of 11 mice each were studied. Animals belonging to the three groups received 5 µg/50 g ptet-mEpoD and 0.5 µg/50 g ptet-tTAk plasmids, while animals from groups 2 and 3 also received 6 and 10 µg/50 g of iCasp9-encoding plasmid, respectively. In order to standardize the amount of plasmid injected to 15.5 µg/50 g, animals from groups 1 and 2 also received naked plasmid (without the FKBP and caspase 9 cDNA). Hematocrit and Epo were determined prior to the plasmid injection.

Four weeks after plasmid injection there was a significant increase in hematocrit in the mice injected with the mEpo plasmid, as shown in Figure 4. The median hematocrit

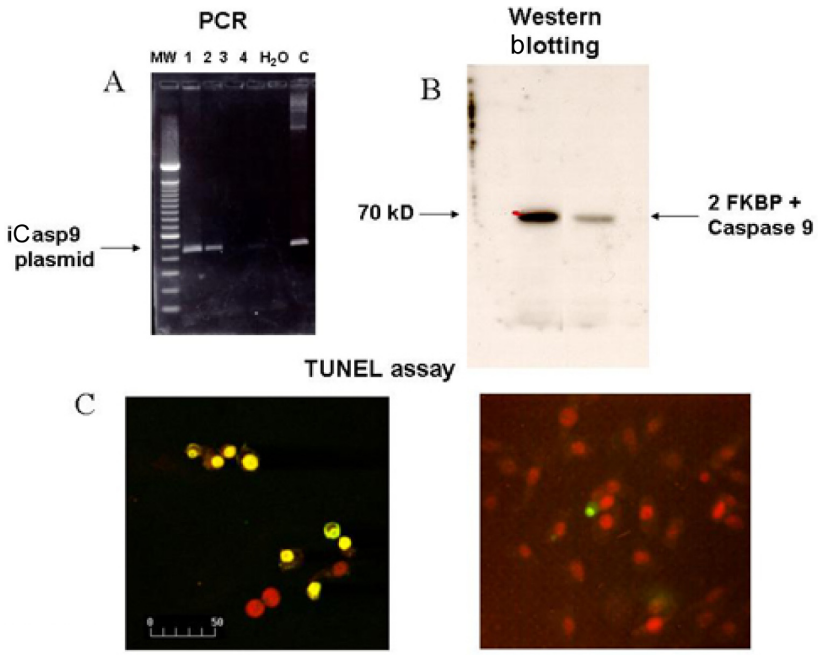

Figure 2. Caspase 9 activity was induced by AP20187 in iCasp9 C2C12 modified cells. *A*, PCR showing the presence of the iCasp9 plasmid in 2 clones (lanes 1, 2) and the absence in 2 negative controls (lanes 3, 4). In the following lanes, water (H_2O) or plasmid (C) was used instead of cell line DNA. MW = ladder 100 bp; *B*, Western blot showing expression of caspase 9 and FK506-binding protein (FKBP) (~70 kD) in clones 1 and 3. Cell extracts were run on 12% SDS-PAGE and immunoblotted with anti-caspase 9 antibody. *C*, TUNEL-positive cells detected after AP20187 treatment (left side). Untreated cells were used as a control (right side).

value increased by 33% above baseline in group 1 (Mann-Whitney test, median difference = 20.0, 95% confidence interval (CI) of the difference = 8.26-25.01; P = 0.0013), while the median hematocrit increased by 36.8% in group 2 (median difference = 17.5, 95%CI of the difference = 10.24-26.59; P = 0.0004), and by 12.8% (median difference = 4.5, 95%CI of the difference = 0.45-12.38; P = 0.0374) in group 3. Thus, we concluded that 6 µg iCasp9 did not reduce Epo vector efficiency, but an opposite result was obtained with 10 µg iCasp9.

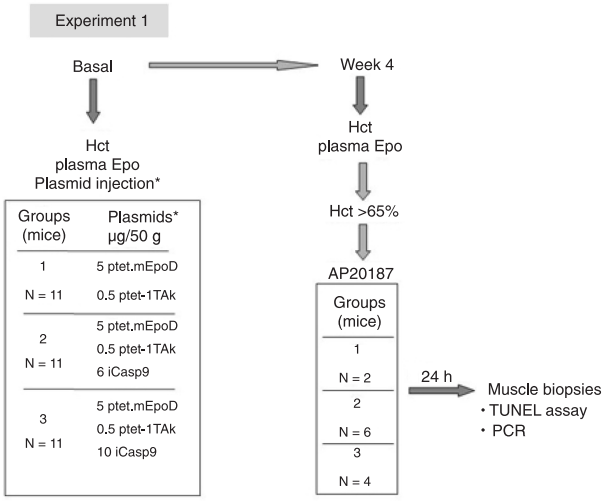

Figure 3. Schematic representation of the *in vivo* studies. Hct - hematocrit; Epo = erythropoietin.

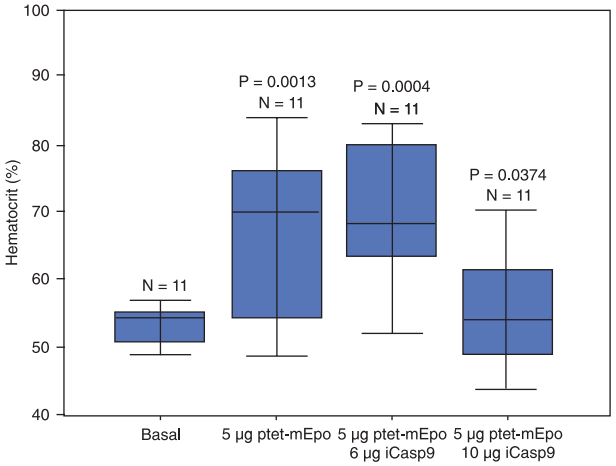

Figure 4. Six micrograms of iCasp 9 plasmid does not reduce erythropoietin (Epo) vector efficiency. Hematocrit before and 4 weeks after intramuscular injection of 5 µg ptet-mEpo/0.5 µg ptet-tTAk plasmids, without or with 6 and 10 µg iCasp9 plasmid. The Wilcoxon-signed rank test was used for statistical analysis.

Detection of TUNEL-positive cells in the injected muscle after AP20187 administration. Two animals from group 1 (injected with 5 µg/50 g ptet-mEpoD and 0.5 µg/50 g ptet-tTAk), 6 animals from group 2 (injected with 5 µg/50 g ptet-mEpoD, 0.5 µg/50 g ptet-tTAk and 6 µg/50 g iCasp9 plasmids), and 4 mice from group 3 (injected with 5 µg/50 g ptet-mEpoD, 0.5 µg/50 g ptet-tTAk and 10 µg/50 g iCasp9 plasmids), which reached hematocrits of more than 65%, were selected for CID treatment and muscle biopsies. Four weeks after plasmid injection, they were treated with AP20187. All animals were sacrificed for muscle studies 24 h after treatment. Plasma Epo was greatly and significantly increased in all of them and the iCasp9 plasmid was detected by PCR in the muscle of the 10 animals injected with it (data not shown). A TUNEL assay showed positive cells in the muscle of 3 of 6 animals from group 2 and in 3 of 4 animals from group 3 (Figure 5). None of the animals from group 1 showed TUNEL-positive cells. Thus, we concluded that 6 µg/50 g iCasp9 is able to trigger apoptosis after AP20187 induction.

Experiment 2

Capacity of iCasp9 and CID to regulate Epo vector expression. Since 10 µg/50 g iCasp9-encoding plasmid decreased the Epo plasmid efficiency, 6 µg/50 g iCasp9-encoding plasmid was injected in the next experiment. The experiment was repeated with two groups of animals. Animals from group 1 (N = 20) were injected with 5 µg/50 g ptet-mEpoD, 0.5 µg/50 g ptet-tTAk plasmids and 6 µg/50 g naked plasmid, and mice from group 2 (N = 36) were injected with 5 µg/50 g ptet-mEpoD, 0.5 µg/50 g ptet-tTAk plasmids and 6 µg/50 g iCasp9 plasmid. Before plasmid injection, the median basal hematocrit was 51% (range: 49 to 55%) and 52% (range: 48 to 58%) for groups 1 and 2, respectively. Basal Epo levels ranged from 2.7 to 4.9 mU/mL (median = 4.6 mU/mL) for group 1 and from 2.4 to 5.3 mU/mL (median = 4.3 mU/mL) for group 2.

In group 1, 4 weeks after plasmid injection, 14 of 20 animals (70%) presented an increase in hematocrit ranging from 65 to 83% (median = 69.24%; Figure 6). All 14 animals received AP20187, 4 weeks after plasmid injection. In order to test the efficiency of the control of Epo secretion by doxycycline, despite co-adjuvant administration of iCasp9 and AP20187, doxycycline was administered as described in Material and Methods to 2 animals for 2 weeks, starting 8 weeks after plasmid injection. The remaining 12 animals sustained their hematocrit above 65% for over 15 weeks (Figure 6) and Epo levels above 90 mU/mL for at least 8 weeks, and reached levels as high as 298 mU/mL (Figure 7).

In group 2, 1 animal died after plasmid injection and electropulse and 2 animals died 10 days later. Interestingly, these 2 animals had a rapid and relatively large increase in hematocrit (data not shown), which reached 74 and 76%, 1 week after plasmid injection. Four weeks

after plasmid injection, hematocrit increased above 65% in 23 of 33 mice (64%; Figure 8A). In these 23 animals, Epo levels ranged from 53 to 234 mU/mL (median = 138 mU/mL; Figure 8B). These 23 animals were selected for AP20187 administration (CID). Two weeks after AP20187 administration, 7 animals (30.44%) presented a significant reduction in hematocrit and plasma Epo (Wilcoxon test). In these 7 animals, hematocrit at week 15 (Figure 9A) ranged from 52 to 65% (median = 54%) and plasma Epo levels (Figure 9B) at week 8, ranged from 2.6 to 46 mU/mL (median = 4.2). Sixteen animals did not respond to the drug (69.56%) and, 4 weeks after CID injection, 6 of them were also selected for doxycycline administration. Animals that did not respond to CID and that were not treated with doxycycline (N = 10) sustained a hematocrit above 78% for more than 15 weeks (Figure 9C), as well as above normal Epo levels (Figure 9D) that ranged from 90 to 251 mU/mL (median = 159 mU /mL).

In summary, experiment 2 demonstrated that the Epo vector efficiently induced a significant and sustained in-

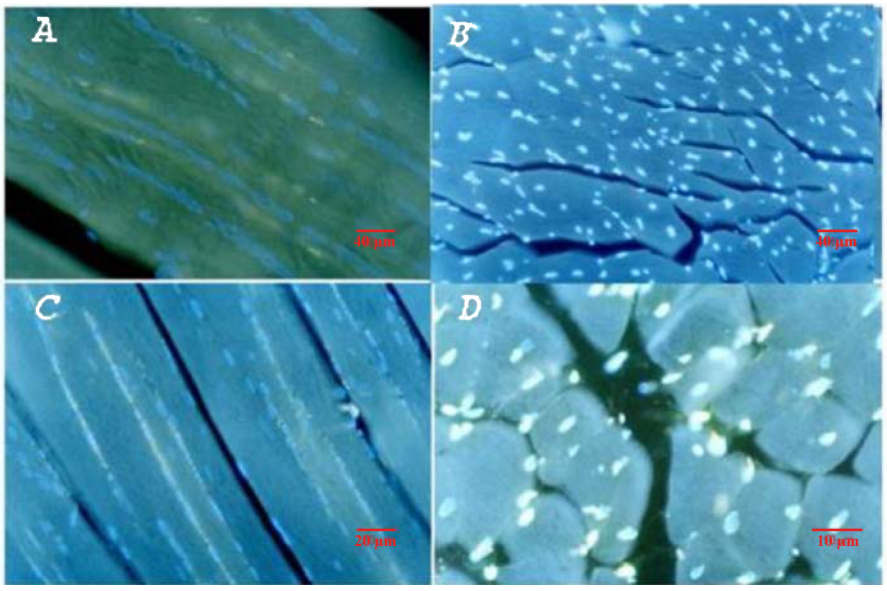

Figure 5. AP20187 induces apoptosis of muscle injected with iCasp9 and 5 µg ptet-mEpo/0.5 µg ptet-tTAk plasmids. *A* and *C* show negative TUNEL reactions in 2 animals not injected with the iCasp9 plasmid. *B* and *D* show positive reactions (indicative of apoptosis) in 2 animals injected with the iCasp9 plasmid. Muscle biopsies were performed 24 h after AP20187 treatment.

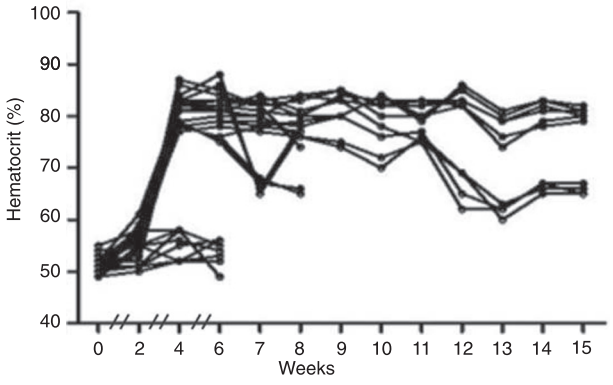

Figure 6. The erythropoietin (Epo) vector induces a large hematocrit increase in 70% of the animals. Hematocrit before and after intramuscular injection of 5 µg ptet-mEpo/0.5 µg ptet-tTAk plasmids showed that 30% of the animals (N = 8) did not present a hematocrit increase of more than 65%. Ten animals sustained the hematocrit above 65% for over 15 weeks.

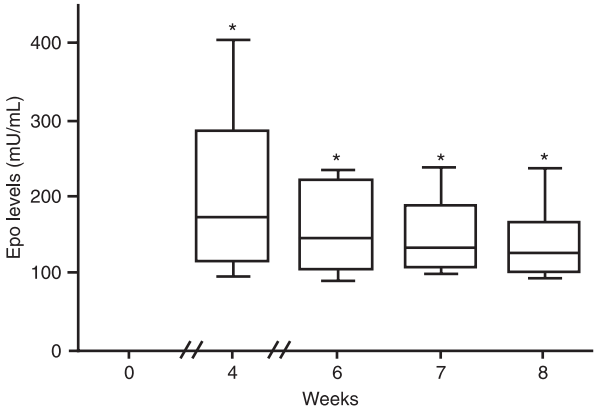

Figure 7. The erythropoietin (Epo) vector induces high levels of plasma Epo. In the animals with hematocrits above 65%, plasma Epo levels (R&D ELISA kit) demonstrated large increases after Epo plasmid injection that were maintained for at least 4 weeks. *P < 0.0001 compared to 0 week (Wilcoxon-signed rank test).

crease in hematocrit and Epo levels even in the presence of iCasp9 plasmid. It also showed that AP20187 induced the iCasp9 system and regulated Epo vector expression in 30% of the animals.

Effect of iCasp9 and CID on the regulation of Epo secretion by doxycycline. The 2 animals from group 1 and the 6 animals from group 2, selected for doxocycline administration as described above, showed a significant reduction

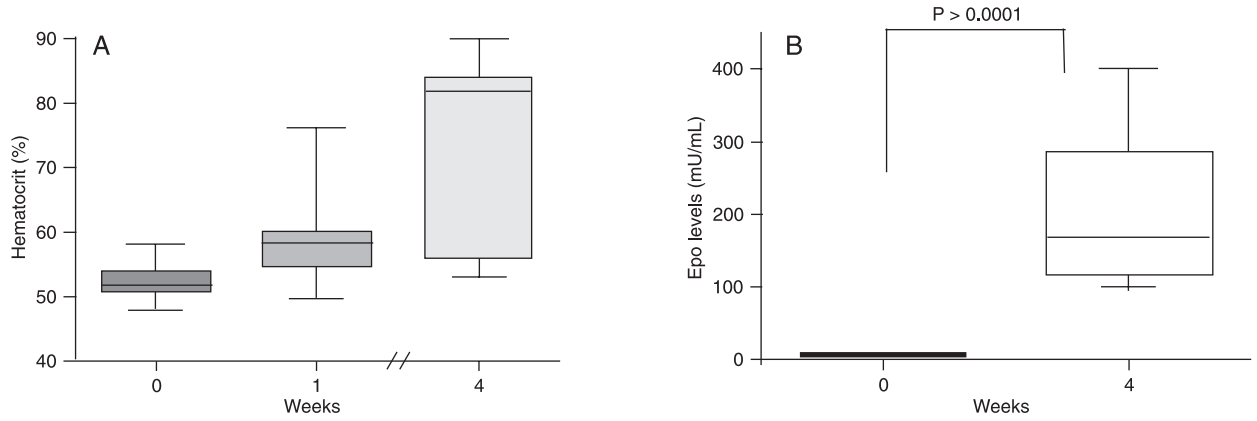

Figure 8. Six micrograms iCasp9 plasmid did not reduce the efficiency of the erythropoietin (Epo) plasmid. *A*, Hematocrit before and 4 weeks after Epo plasmid injection in animals that also received the iCasp9 plasmid; 70% (N = 23) of the animals obtained a hematocrit increase of more than 65%. *B*, Plasma Epo levels were significantly increased in these 23 animals, 4 weeks after plasmid injection (Wilcoxon-signed rank test).

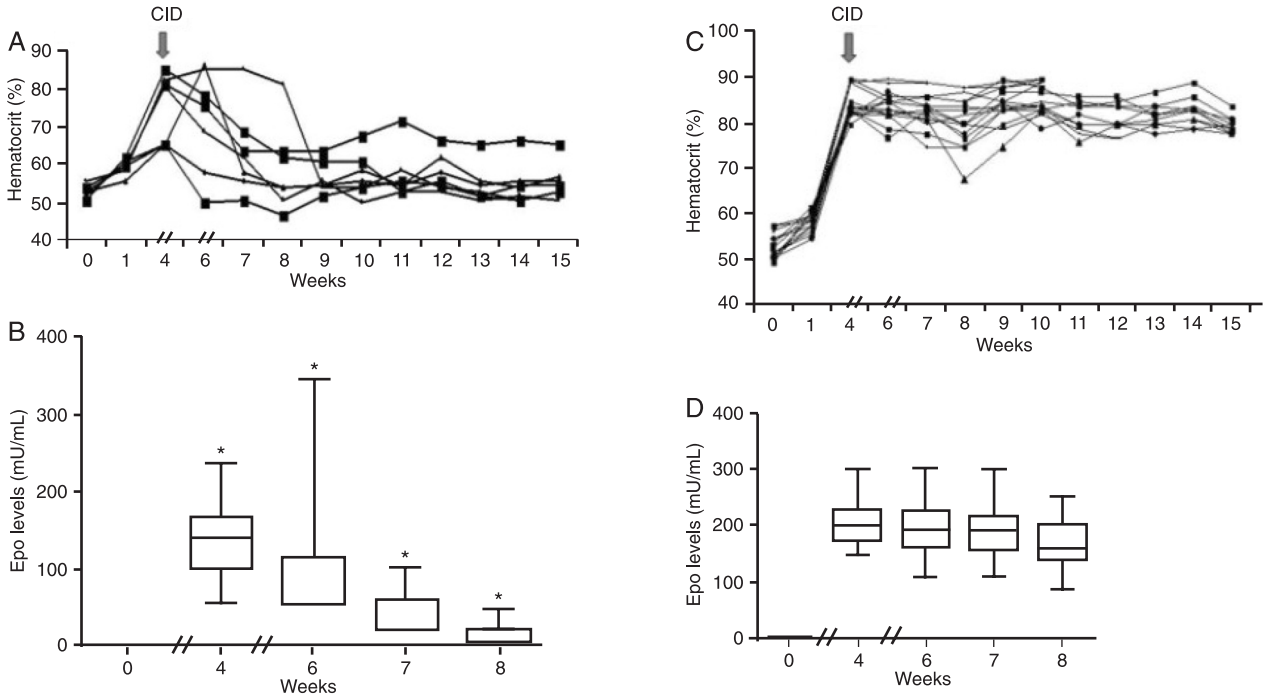

Figure 9. The iCasp9 system was induced efficiently by AP20187 in 30% of the animals. Twenty-three mice received 5 µg erythropoietin (Epo) vector/50 g, 0.5 µg ptet-tTAk/50 g and 600 µg/50 g iCasp9 plasmids. Two weeks after AP20187 administration, 7 of them (30.4%) showed a significant reduction in hematocrit (A) and Epo levels (B) (*P < 0.03, Wilcoxon-signed rank test). Sixteen animals (69.6%) did not respond to chemically induced dimerization (CID). Six of these were selected for doxycycline administration and the remainder (N = 10) sustained their hematocrit above 78% for more than 15 weeks (C), as well as high levels of Epo (D).

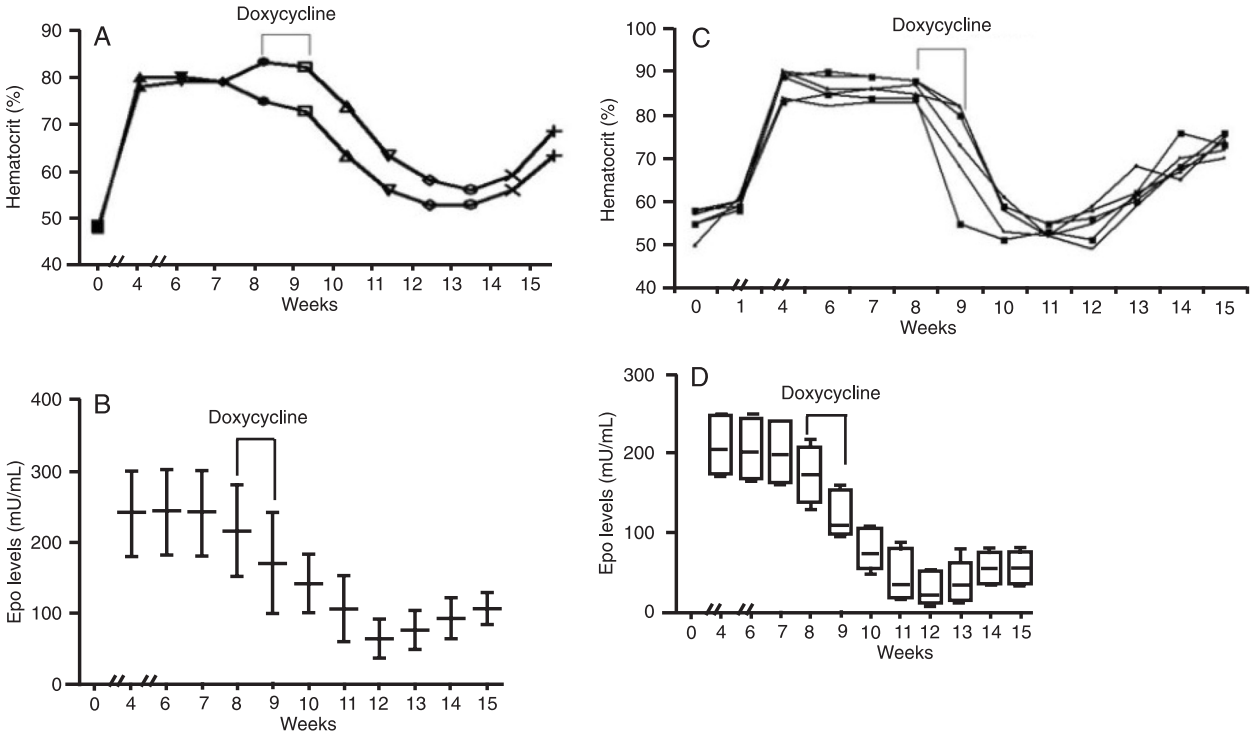

Figure 10. The iCasp9 system permitted efficient induction of the Tet-off system by doxycycline. Doxycycline was administered in drinking water for two weeks to 2 animals injected with 5 µg Epo/0.5 µg ptet-tTAk (group 1) and to 6 animals also injected with 6 µg iCasp9 plasmid (group 2). Similar reductions in hematocrit and Epo levels were observed when comparing group 1 (A, B) and group 2 (C, D). The data for the 2 mice in Panel A and the 6 mice in Panel C are presented with the same symbols.

in hematocrit and plasma Epo levels, 1 week after the beginning of doxycycline administration. Lower hematocrit and plasma Epo levels were reached after 3 weeks. No significant difference in hematocrit (Figure 10A and C) or plasma Epo levels (Figure 10B and D) was detected when comparing groups 1 and 2. Thus, we concluded that doxycycline efficiently regulated Epo secretion in the presence of the iCasp9 plasmid and AP20187 administration.

Detection of the iCasp9 vector in the injected muscle. PCR for the detection of the iCasp9 plasmid was performed in all mice from group 2, except for the 3 mice that had died earlier. The iCasp9 plasmid was not detected in 10 animals, and none of these responded to CID administration. The plasmid was detected in the remaining 23 animals, including 6 mice that did not respond to AP20187 administration (data not shown). Thus, we concluded that the absence of a response to CID could be related to a low copy number of the iCasp9 vector.

Discussion

Therapeutic proteins may be delivered by gene therapy. The most promising results in this field have arisen from the treatment of hemophilia B patients with adeno-associated virus vectors expressing factor IX (11,12). Increasing evidence emerging from *in vivo* studies demonstrates that erythropoietin is another protein that may be used in gene therapy with benefits to a large number of patients. The high cost of recombinant hEpo restricts its correct use, since hospital administrators only allow administration of hEpo when severe anemia is already established. In most developing countries, hEpo, at low doses, is approved only for renal failure, preventing the use of higher doses of hEpo to treat diseases in which Epo resistance is common (such as myelodysplasia, hemoglobinopathies or cancer). Thus, gene therapy may reduce the cost of treatment and return the therapeutic decision to doctors and patients, rather than to payers, as already mentioned by Lippin et al. (26).

Most studies using viral or non-viral vectors carried out in mice or non-human primates, and recently in patients with chronic renal failure, have shown that erythropoetin gene therapy leads to therapeutically relevant levels of the Epo hormone (26,30). However, an undesirable side effect of this therapy could be polycythemia and the risk of thrombosis (19,20). Autoimmune anemia has also been described due to the appearance of neutralizing antibodies against endogenous Epo (20,31). Preliminary *in vivo* studies of the regulation of Epo secretion by oxygen seem promising, but few studies have been carried out in this field (28,32,33). Regulation of Epo secretion by doxycycline is

possible and seems efficient (19,25) and has been observed in non-human primates when the immune system is not mobilized against the tetracycline-dependent transactivator (34). However, if a definitive gene switch is necessary to repress expression, the patients would need to be continually on medication. Thus, a fail-safe method to switch off the gene therapy would be to destroy the modified cells by triggering apoptosis.

Besides the expected low efficiency of gene therapy using non-viral vectors, these do not integrate in the genome and there is extremely little, if any, risk of cancer using this strategy. Moreover, viral vectors have also been associated with low levels of protein expression, as observed in gene therapy for hemophilia (35) or, more recently, for chronic renal failure (26), probably due to immunogenic reactions against the gene vehicle. Thus, in our study we decided to test induction of apoptosis for safety in gene therapy, using non-viral vectors as a platform. Erythropoietin was used in this study and the therapeutic gene and safety measure used was the inducible caspase 9 gene (iCasp9), which was regulated by the AP20187 CID. Compared to caspases 3 and 7, iCasp9 is expressed at sufficient levels to confer sensitivity to CID (36,37). This system has low potential immunogenicity and the CID has no side effects (38). Therefore, pharmacological studies of AP20187 suggest widespread bioavailability.

In order to obtain sustained and regulated levels of Epo, in our *in vivo* studies we electro-transferred into mouse skeletal muscle the already successfully tested dimeric murine Epo-Epo plasmid containing the tetO element and a plasmid encoding the tetracycline-controlled transactivator (25,29). This system works very well in mice and doxycycline efficiently controlled the secretion of dimeric Epo without inducing antibody formation. Therefore, high efficiency of gene transfer by electric pulse has been demonstrated *in vivo* (39,40). In our studies, the efficiency of the mEpoD plasmid in animals receiving or not 6 µg iCasp 9 plasmid was observed in about 70% of the animals of both groups and was sustained for at least 15 weeks. However, no increase in plasma Epo levels or hematocrit was observed in 30% of the animals from the two groups. This failure may be due to several factors, including the inefficient transfer of the plasmid to the muscle cells, failure in the expression of the protein or even an immune reaction caused by the electropulse (25,39,40).

In the present study, we also observed a low response to the Epo vector in mice receiving 10 µg iCasp9, suggesting that CID-independent basal toxicity may have reduced survival of Epo plasmid-transduced cells. In fact, iCasp9 has a much lower basal activity than FAS and iCaspase8, but always maintains a low basal activity due to overexpression (3). However, cells that overexpress iCasp9 at toxic levels will be destroyed early due to autoactivation of caspase 9, leaving a stable cohort of cells that are still sensitive to CIDs (9). On the other hand, any cells expressing levels of iCasp9 too low for activation will probably express irrelevant levels of Epo. Thus, there should be a "therapeutic window" in which iCasp9 could be useful.

The gene expression control by the EPO doxocycline induction system used in this study has been well documented in the literature (28) and was reproduced here in 2 animals. We also showed that the efficiency of doxycycline administration in controlling Epo secretion was similar in the group of animals receiving the iCasp9 vector, according to hematocrit and plasma Epo measurements. Thus, we concluded that iCasp9 may be used as a safety mechanism in combination with other vectors, without interfering with gene therapy efficiency.

Regarding the efficiency of the iCasp9 plasmid in inducing apoptosis, 30% of the animals that received the iCasp9 vector responded to AP20187 administration. In these animals, the iCasp9 vector was very efficient in inducing apoptosis since Epo levels and hematocrit reached basal levels in less than 4 and 6 weeks, respectively, after CID administration. TUNEL-positive cells were detected in the muscle of two animals injected with the iCasp9 plasmid and treated with CID.

Failure in efficiently capturing the iCasp9 plasmid by the muscle cells, a low copy number of the vector or inadequate CID concentration or distribution may have contributed to the low efficiency of the system in these initial studies. The activation of iCaspase9 by a synthetic dimerizing drug is a strategy that can be used to destroy transformed cells and to control undesirable side effects of gene therapy, although more studies are necessary to improve this technique.

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