Monitoring Gene Therapy by External Imaging of mRNA: Pilot Study on Murine Erythropoietin

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Abstract: Gene therapy is anticipated as being an important medical development. Essential to its effectiveness is the appropriate activity (protein expression) in the expected target cells. A noninvasive diagnostic procedure of successful gene expression will be of paramount importance to validate its use or its misuse (eg, sports gene doping). Externally detectable labeled oligonucleotide hybridizing with the messenger RNA generated by the transferred gene has been proposed as a possibility to monitor successful gene therapy. The authors selected the erythropoietin gene (*Epo*) for a pilot study on erythropoietin protein expression in mouse muscle. Oligonucleotides of peptide nucleic acid (PNA) type capable of antisense binding to unique murine *Epo*-mRNA sequences were synthesized by solid phase methods, and elongated at the N-terminus with the HIV Tat (48–60) cell penetrating peptide. They were labeled with fluorescence and radioactive tags to verify penetration and longer half-life properties in *Epo* gene transfected C2C12 mouse muscle cells as compared with corresponding wild-type cells. Downregulation of newly expressed erythropoietin protein in such cells additionally confirmed the penetration and hybridizing properties of the selected labeled oligonucleotide. ^{123}I-labeled Tat-PNAs were intravenously injected into mice that had previously received the *Epo* gene into the right tibialis muscle by DNA electrotransfer. Preferential accumulation of radioactivity in the transferred limb as compared with the contralateral limb was ascertained, especially for ^{123}I-Tat-CTA CGT AGA CCA CT (labeled Tat-PNA 1). This study provides experimental data to support the potential use of external noninvasive image detection to monitor gene therapy. The extension of the approach to more sensitive methods for whole-body external detection such as positron emission tomography appears feasible.

Key Words: gene therapy, gene doping, gene expression, detection, peptide nucleic acid, antisense

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INTRODUCTION

Gene therapy is increasingly being viewed as a new branch of regular medicine. Essential to its effectiveness is the appropriate activity (protein expression) in the expected target cells. A noninvasive diagnostic procedure to verify such an effect will be of paramount importance to monitor its use[1,2] and its misuse.[3] Among the latter, the injection of a plasmid DNA expressing a prohibited substance in the field of sport should be considered.[4] This phenomenon raises an important issue in the area of doping control related to the existence of reliable methods for the detection of possible gene transfer abuse.[5]

Monitoring gene expression by noninvasive imaging techniques has already been proposed[6] in medicine as an alternative to invasive approaches (ie, using biopsies). Several methods for external imaging of gene expression have been proposed, but positron emission tomography and single photon emission computerized tomography technologies appear to be the most sensitive tools in this regard.[7,8] Besides the detection of the expressed protein, it is possible to image in vivo the presence of a given messenger (m)RNA by the use of labeled antisense molecules that hybridize to a specific nucleotide sequence within the target mRNA.[9] Antisense oligonucleotides have been used and shown to be well tolerated by animals and patients with low toxicity and relatively low cost. However, the limitations are clear; phosphodiester oligonucleotides are rapidly degraded in vivo[10] and although phosporothioate oligonucleotides partially correct this degradation, they are avidly bound by serum proteins, retarding uptake into tissues.[11]

Another class of antisense molecules are the oligonucleotide analogs, peptide nucleic acids (PNA).[12,13] Here, the negatively charged sugar–phosphate backbone has been replaced by a neutral backbone consisting of repeating N-(2-aminoethyl)glycine units linked by amide bonds with the common nucleic acid bases attached through a carbonyl methylene linker. PNA oligomers recognize and bind to a specific DNA or RNA strand with high affinity and selectivity.[14] Their high chemical and in vivo stability (resistant to both nucleases and proteases) and low binding to serum proteins make them good candidates as therapeutic agents, diagnostic tools, and probes in molecular biology.[15–17] A limitation could arise from observations that free PNAs are poorly transported across biologic membranes.[18,19] However, chemical modifications based on covalently linked

cell-penetrating peptides have been used successfully to deliver PNAs into cells.[20,21]

In the present research, we aim to evaluate the feasibility of PNA-antisense noninvasive imaging of an mRNA giving rise to ectopic expression of doping hormones after gene transfer, taking erythropoietin gene (*Epo*) and erythropoietin protein (EPO) as the studied targets.

METHODS

Peptide Nucleic Acid Sequences

Minimal length nucleotide sequences from murine *Epo* cDNA[22] (GeneBank Accession no. NM_007942) with no homology to the rest of the murine genome were selected by means of appropriate software [BLAST (NCBI) and FASTA SEEK]. Three independent sequences of 14 nucleotides were identified: 1) AGU GGU CUA CGU AG (positions 330–343); 2) GGG UCU ACG CCA AC (positions 460–473); and 3) CGA CAG UCG AGU UC (positions 394–407). To prioritize one of these sequences, a search for theoretical three-dimensional structures was performed by means of appropriate software packages [(RNA fold, Vienna RNA package); (RNA structure program); (Gene Quest, DNAStar Inc., Madison, WI)]. The antisense to 1) complementary sequence CTA CGT AGA CCA CT (subsequently named PNA 1) was chosen based on putative higher hybridization access (less internal pairing). The antisense sequence to 2) GTT GGC GTA GAC CC (PNA 2) and one-point mutation to PNA 2 (GTT GGC CTA GAC CC, mutation underlined; mutant PNA 2) were also selected as controls for some experiments.

Synthetic Tat-PNA Chimera

Tert-butoxy-carbonyl (t-Boc)-PNA monomers were purchased from Applied Biosystems (Foster City, CA); Boc- and fluorenylmethoxycarbonyl (Fmoc)-protected amino acids were from Senn Chemicals (Dielsdorf, Switzerland). p-Methylbenzhydrylamine resin was from Novabiochem (Läufelfingen, Switzerland). O-(7-azabenzotriazole-1-yl)-N, N,N′N′-3-tetramethyluronium hexafluorophosphate (HATU) was from GenScript (Piscataway, NJ). Peptide synthesis solvents (dimethylformamide, dichloromethane) and high-performance liquid chromatography-grade acetonitrile for purification were from SDS (Peypin, France) as was the trifluoroacetic acid. Trifluoromethane sulfonic acid and other reagents were from Sigma-Aldrich (Madrid, Spain).

The general structure of the Tat peptide-PNA construct is shown on Figure 1A. The construct was assembled by solid phase methods[12,23,24] on p-methylbenzhydrylamine polystyrene resin using a mixed Boc/Fmoc protection scheme. First, the C-terminal Cys-Gly dipeptides, and the ensuing PNA monomers, were incorporated manually by Boc-based chemistry. After 14 PNA synthetic cycles, the Boc protecting group was removed with trifluoroacetic acid and the first residue (Gln) of the Tat(48–60) peptide moiety was incorporated using HATU for activation. The Fmoc-Gln-PNA-CysGly-resin was then placed in a peptide synthesizer (Applied Biosystems 433) and the remaining residues of the Tat sequence (GRKKRRQRRPP) were incorporated by optimized Fmoc/t-Bu peptide synthesis protocols. After the completion of the chain assembly, the protected Tat-PNA hybrids were fully deprotected and cleaved from the resin by treatment with trifluoromethane sulfonic acid/trifluoroacetic acid/m-cresol for 90 minutes at 25°C. The products were isolated by precipitation with chilled tert-butyl methyl ether and centrifugation, purified by preparative reversed phase high-performance liquid chromatography, and further characterized by MALDI-TOF (Matrix-assisted laser desorption ionization, time-of-flight)[4] mass spectrometry (Voyager DE-STR; Applied Biosystems) using sinapinic acid as matrix. Quantitation was performed by ultraviolet spectrophotometry at 260 nm. Complete details on the synthesis and characterization of the construct will be given elsewhere (de la Torre et al, unpublished data).

Labeling

Fluorescent Labeling

Purified Tat-PNA constructs were dissolved in 50 mM Tris buffer, pH 7.5, and reacted with thiol-reactive fluorescein-5-maleimide (Molecular Probes, Leiden, The Netherlands). The conjugates were separated from excess reagent by gel filtration on a Sephadex G-25 NAP10 column (GE Healthcare) eluted with water. The labeled products were lyophilized.

Radioactive Labeling

Synthesis of the radioactive precursor [N-(4-[^{123}I]iodobenzyl)-2-bromoacetamide] was carried out as described.[25] The ^{123}I-labeled version of Tat-PNA was prepared by reaction of the corresponding Tat-PNA construct with the radioactive precursor in 1 M Tris (pH 8.5, 70°C for 25 minutes). Labeled ^{123}I-Tat-PNAs were purified (greater than 98%) by semipreparative reversed phase high-performance liquid chromatography.

Generation of Stable C2C12 Cells Expressing Murine Erythropoietin Protein

C2C12 mouse myoblast cells were obtained from the European collection of cell cultures and cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL), streptomycin (100 μg/mL) and 2 mM glutamine (Invitrogen Corp., Paisley, UK). Cells were transfected with 10 μg of the pcDNA.3m*Epo* expression vector[26] using Superfect transfection reagent (Qiagen, GmbH, Hilden, Germany) following the manufacturer's instructions. Cells were plated in 100-mm cell culture dishes and subjected to G418 (Invitrogen) selection (800 μg/mL). Pools and individual clones of resistant cells were grown and assayed for murine EPO production by specific enzyme-linked immunosorbent assay (Quantikine R&D) for mice.

Tat-PNA Incorporation Into Cells

The penetration of fluorescent-labeled naked PNAs (PNA 1 and mutant PNA 2) into C2C12 cells was studied under different conditions: 1) direct incubation of PNAs alone; 2) PNAs in the presence of classical cationic lipofectamine DNA transfection medium; 3) PNAs preincubated with their complementary oligonucleotide[24]; and 4) PNA preincubated with carrier peptides Pep-2[21] and Tat. In all of these conditions, the number of cells showing incorporated fluorescence never

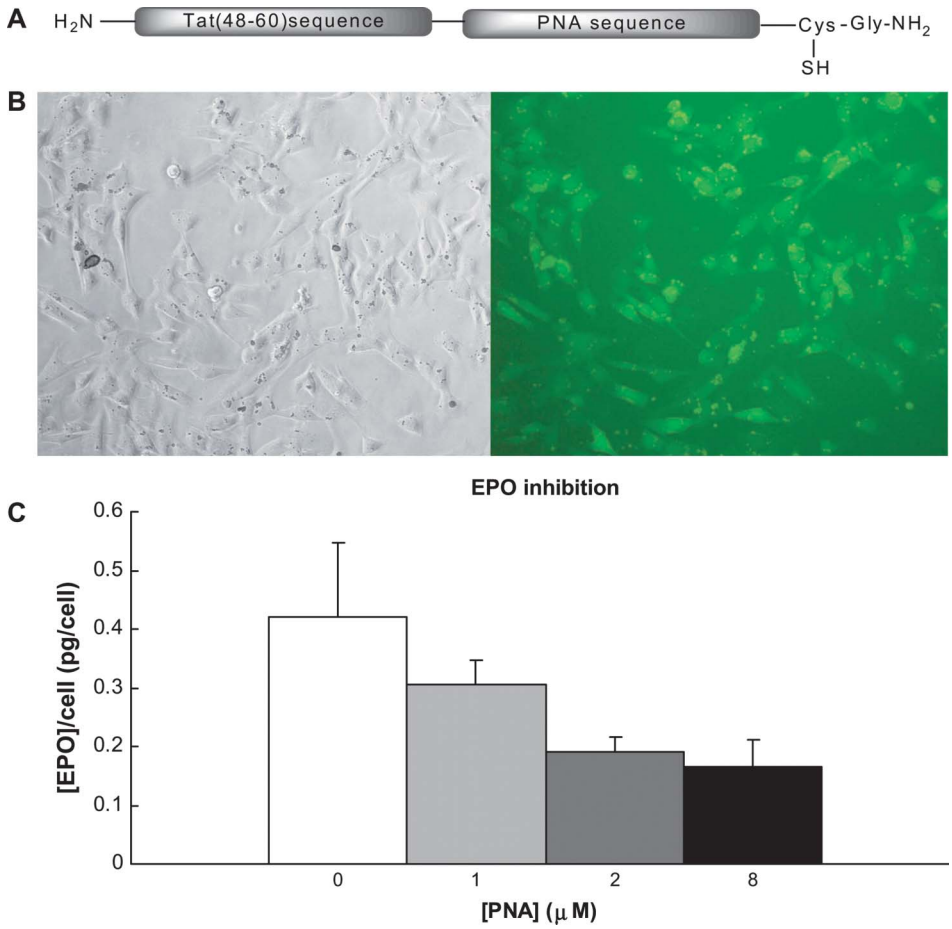

FIGURE 1. Fluorescently labeled Tat-PNA and its effects on C2C12 *Epo* gene-transfected cells. (A) General structure of Tat-PNA chimeric construct. Fluorescence was attached to the sulfur atom (B) Tat-PNA delivery into cells, which were incubated with fluorescently labeled Tat-PNA 1 for 16 hours and extensively washed until observation. Images were captured in living cells with an inverted Leica microscope (400×): (left) phase contrast; (right) fluorescent. (C) In vitro effects of Tat-PNA 1 on murine erythropoietin protein production. Increasing concentrations of labeled-TAT-PNA 1 (none, 1, 2, and 8 μM) were incubated with transfected cells. Erythropoietin protein concentration was measured in conditioned media after 48 hours. Values are mean ± standard error of mean (n = 3).

exceeded 30% and the strategy was further shifted toward the Tat-penetrating peptide covalently bound to the PNAs (Tat-PNAs).

The following experiments were carried out both in C2C12 wild-type and EPO expressing cells: 1) verification: after incubation for 16 hours with fluorescent-labeled Tat-PNAs (PNA 1 and mutant PNA 2), fluorescence in the cells was verified by microscope imaging and EPO concentrations in new media were measured at different times thereafter; 2) kinetics: cells were incubated with ^{123}I-labeled Tat-PNA 1 and radioactivity retained in the cells was counted at different times; and 3) elimination: cells were incubated for 3 hours with ^{123}I-labeled Tat-PNA 1. After media replacement, radioactivity remaining in the cells and concentration of EPO in supernatants were measured at different times.

Gene Transfer to Mice

Animals

Six-week-old male BALB/c mice (20–25 g) (Charles River, Lyon, France) were anesthetized intraperitoneally with a dose of 0.02 mL/g body weight of 1.2% Avertin solution (2,2,2-tribromoethanol in tert-amylalcohol). The legs were shaved and moistened with 70% ethanol. Plasmid pcDNA.3m *Epo* was prepared with the EndoFree Plasmid Giga Kit (Qiagen) and formulated with the SP1017 nonionic carrier as previously described.[27] Fifty micrograms of DNA was injected into the tibialis anterior muscle of the right lower extremity with a 28-gauge needle inserted in a proximal to distal direction. The left leg received only the nonionic carrier. The injected volume was 50 μL. Mice were fed ad libitum and maintained under a 12-hour light–dark cycle. Animal procedures were performed in accordance with recommendations for the proper care and use of laboratory animals and the study received institutional ethics approval.

Electroporation

After the intramuscular injection of plasmid DNA, a conductive gel was applied to the shaved leg to ensure electrical contact and an electrical field was applied to the area around the injection. Muscles were held by caliper electrodes composed of two 1.5-cm^2 steel plates and electric pulses were delivered to the muscle by a pulse generator (BTX ECM830 electroporator; Genetronics Inc., San Diego, CA). Eight 20-ms pulses at a frequency of 2 Hz at an output voltage of 175 V/cm were used. To improve plasmid DNA diffusion, 25 units of bovine hyaluronidase (Sigma Aldrich) in 25 μL saline solution were injected into the muscle 2 hours before the administration of the plasmid DNA and electroporation.

On days 2, 7, and 14 thereafter, EPO in blood plasma was measured by enzyme-linked immunosorbent assay (see

previously) and hematocrit in whole blood by microcapillary centrifugation.[28,29]

^{123}I In Vivo Imaging

An experiment by single photon emission plannar gammagraphy with three mice, which were injected with ^{123}I-labeled Tat-PNA 1 (one gene-transferred and one non-gene-transferred control mice) and ^{123}I-labeled Tat-PNA 2 (one gene-transferred mouse), respectively, was carried out. Animals were anesthetized with isoflurane and injected with 10 to 12 MBq in the tail vein. The rodents were scanned in a Siemens Orbiter 750 gamma camera, over 15 minutes, using a pinhole collimator of 6 mm and 60° of aperture. All the experiments were carried out using an energy window of 20% around 159 KeV. Semiquantitative tracer uptake in the transferred leg of each animal was obtained by subtracting the measured activity of the right leg from that of the left one at equivalent points in a virtual line throughout both limbs.

RESULTS

Tandem Tat-PNA Construct

The peptide(Tat)-PNA hybrids (Fig. 1A) were assembled by optimized stepwise solid phase methods using Boc and Fmoc chemistries for the PNAs and peptide moieties, respectively. The completed sequences were cleaved from the resin and fully deprotected (amino acid side chains and nucleobase amino groups) in a single step by acidolysis. After LC[5] purification, the target products were satisfactorily obtained and their identity and homogeneity was verified by MALDI-TOF mass spectrometry and analytic high-performance liquid chromatography, respectively.

Tat-PNA Incorporation Into C2C12 Cells and Effects

High transfection efficiency (more than 90% of cells showing fluorescence incorporated) of Tat covalently bound to the PNAs (Tat-PNAs) into wild-type and transfected C2C12 cells was achieved (see Fig. 1B for Tat-PNA 1; similar results were obtained for mutant Tat-PNA 2). Fluorescently labeled Tat-PNAs molecules were microscopically observed mainly in the cytoplasm of the treated cells.

The consequences of Tat-PNAs cell penetration on EPO production as an indirect measure of Tat-PNAs hybridizing to *Epo* mRNA were further studied. The results of EPO concentration measured by enzyme-linked immunosorbent assay (Fig. 1C) suggested a trend ($P = 0.12$ at 2 μM as compared with 0 μM) toward a dose-dependent partial inhibition of the EPO production with Tat-PNA 1. No effect was observed for mutant Tat-PNA 2. These results, although not statistically significant, suggested that after Tat-PNA 1 entering the cells, hybridization of PNA 1 with *Epo* mRNA in the expressing cells might have occurred. Additional information on strong hybridization between PNA 1 and nucleic acids was available from fusion curves; mixing PNA 1-Cys-Gly with an equimolar amount of complementary oligodesoxyribonucleotide had produced a strong hyperchromic effect with no inflexion point observed even up to 80°C, suggesting a high stability of the corresponding duplexes.

Tat-PNA Elimination From Cells

To gain insight into potential differentiation between transfected and nontransfected cells regarding further elimination of labeled Tat-PNA 1 previously incorporated into cells, Tat-PNA 1 labeled with a radioactive halogen atom (^{123}iodine) was used to best mimic the future in vivo situation.

On incubation with ^{123}iodine-Tat-PNA 1 transfected cells were able to incorporate a statistically ($P < 0.05$) larger amount of radioactive labeling than wild-type cells. Differential accumulation was observed as early as 30 minutes after starting incubation and continued over the 7-hour period studied (Fig. 2A). To verify if the accumulated radioactivity could leave the cells later, sets of transfected and nontransfected cells after 3 hours incubation with ^{123}iodine-Tat-PNA 1 were placed in fresh nonradioactive medium and the radioactivity remaining in the cell was measured at different times (Fig. 2B). Both transfected and nontransfected cells displayed rather slow radioactive clearance rates, but residual radioactivity up to the 8-hour exposure to fresh medium[6] was statistically higher in transfected cells. Those transfected cells produced a lower amount of EPO (statistically significant $P < 0.05$) than a parallel group of transfected cells treated the same way but not exposed to the labeled Tat-PNA, confirming the inhibitory effect on EPO expression (Fig. 2C).

In Vivo Study

A mouse model of intramuscular *Epo* gene transfer was generated by DNA electroporation.[28,29] The effectiveness of *Epo* electrogene transfer to mice was assessed by the measurement of circulating EPO concentrations and hematocrit. EPO concentrations increased sharply with a subsequent decline after 1 week that remained higher than control mice and persisted up to 14 days (Fig. 3A). Hematocrit increased steadily during first days, reaching an apparent stabilization in 10 to 15 days (Fig. 3B).

The scintigraphy for the gene-transferred mouse injected with ^{123}I-Tat-PNA 1 is shown in Figure 3C. A semiquantitative estimation is obtained by the difference in activity observed along a virtual line drawn through the right (gene transferred in noncontrol animals) and the contralateral (nongene-transferred) left limbs. The graphic display of differential radioactivity for the three mice injected with labeled Tat-PNAs are presented in Figure 3D. Clear accumulation of radioactivity is objectively observed in the right limb of the gene-transferred animals without accumulation effect in the nongene-transferred mouse, thus indicating a substantial increase in radioactivity in the gene-transferred limbs, especially for labeled Tat-PNA 1.

DISCUSSION

In this work, our aim was to investigate the feasibility of monitoring gene therapy by in vivo external imaging in a mouse model. As a first step toward this goal, a 14 nucelotide sequence from the murine *Epo* mRNA[22] with no homology to the rest of the murine genome was selected and an antisense

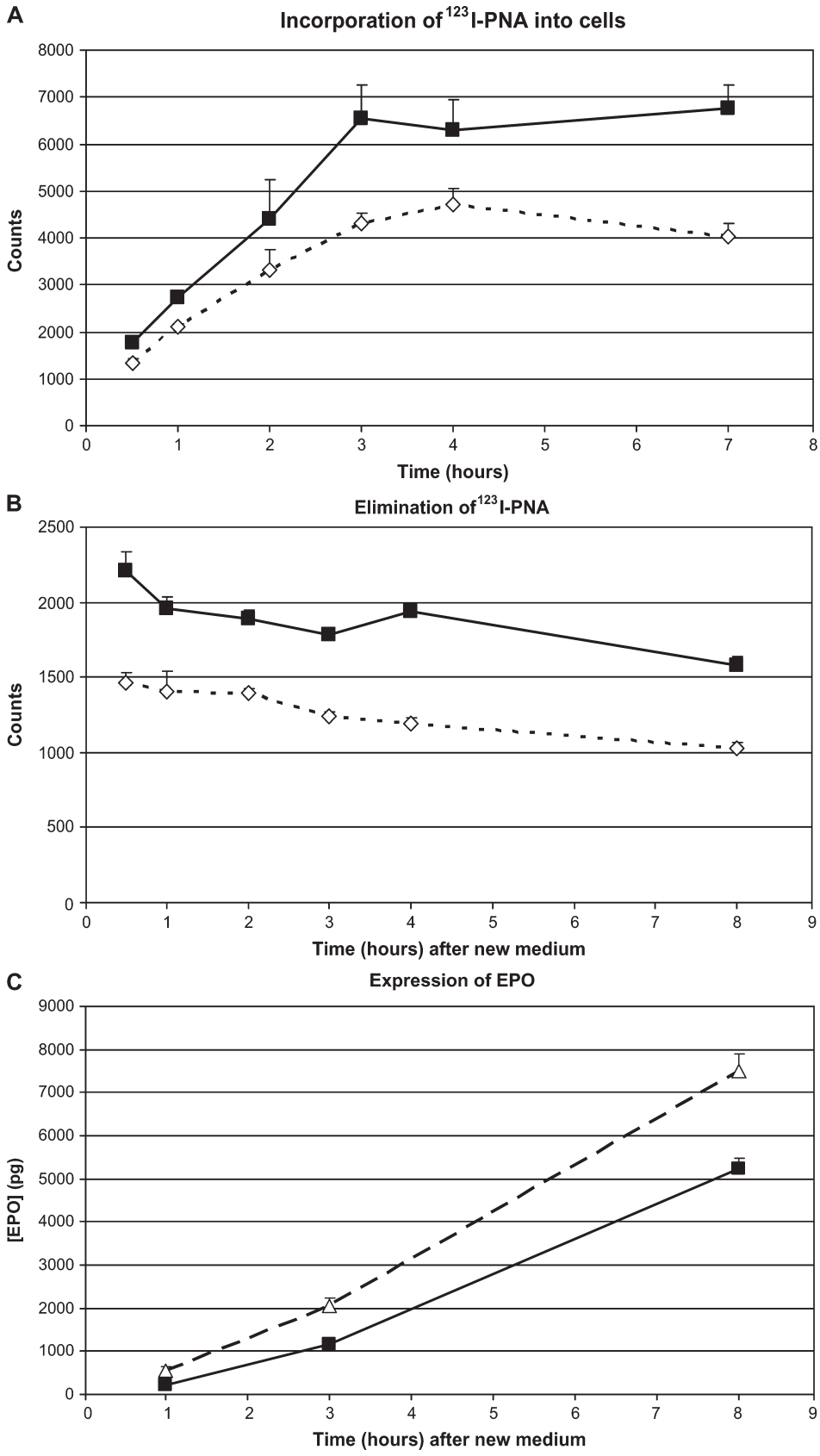

FIGURE 2. Kinetics of ^{123}I-Tat-PNA 1 disposition in C2C12 cells. (A) Measurement of radioactivity inside transfected (closed squares) and nontransfected control cells (open diamonds) incubated with ^{123}I-Tat-PNA 1 for up to 7 hours. (B) Measurement of radioactivity inside transfected (closed squares) and nontransfected control cells (open diamonds) previously incubated with ^{123}I-Tat-PNA 1 for 3 hours and exposed to new nonradioactive medium. (C) Expression of erythropoietin protein in cells previously incubated with ^{123}I-Tat-PNA 1 for 3 hours and exposed to new nonradioactive media (same cells as in B; closed squares) compared with transfected cells not incubated with the labeled Tat-PNA construct (open triangles).

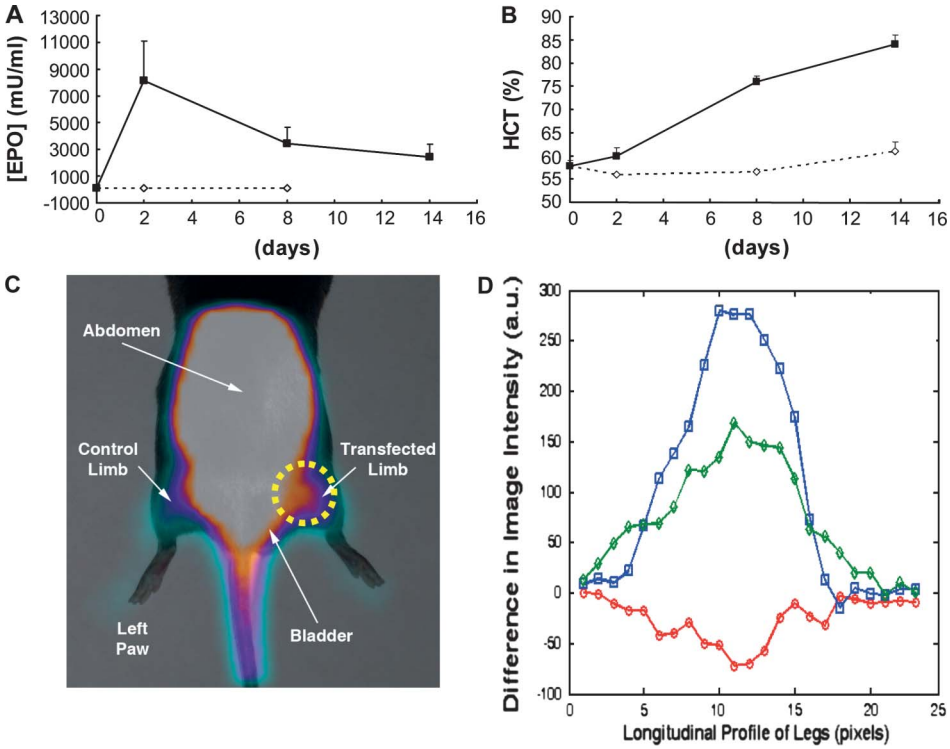

FIGURE 3. In vivo study with mice injected in the right rear limb with pcDNA.3m*Epo* formulated with SP1017 followed by electroporation. (A) Time course (mean ± standard error of mean, n = 3) of circulating erythropoietin protein serum concentrations (closed squares) compared with nongene-transferred animals (open diamonds). (B) Time course of hematocrit in the same animals. (C) Planar gammagraphic image of one gene-transferred mouse after injection with ^{123}I-Tat-PNA 1. The experiment was carried out 15 days after gene transfection. The image was manually overlaid on a photographic picture of a mouse for better localization of the activation. (D) Differences in image intensities between right and left limb in two gene-transferred animals injected with ^{123}I-Tat-PNA 1 (open squares) and ^{123}I-Tat-PNA 2 (open diamonds), respectively, and one control (nongene-transferred) animal injected with ^{123}I-Tat-PNA 1 (open circles).

complementary sequence was built in PNA form. To facilitate the delivery of this PNA into target cells, a chimeric construct consisting (from N- to C-terminus) of a cell-penetrating Tat(48–60) peptide sequence followed by the PNA sequence and by a Cys residue for labeling purposes was prepared. The construct was efficiently assembled by a combination of manual Boc (PNA segment) and automated Fmoc (Tat segment) solid phase synthesis methods. The C-terminal Cys residue allowed chemospecific labeling of either the PNA moiety alone or the whole Tat-PNA construct using either fluorescent dyes or radioactive probes. In the latter, the (N-4-halobenzyl)-2-bromoacetamide auxiliary unit attached to the construct through the Cys residue opens a pathway for alternative incorporation of halogen atoms such as ^{18}F suitable for positron emission tomography detectability.[30,31]

Covalently linked Tat-PNAs substantially entered the cells.[20] The presence of strong fluorescence after suitable in vitro incubation of cells with the fluorescent Tat-PNA construct indicated the suitability of the approach. Also, the trend toward partial inhibition in an apparent dose-dependent manner of the production of EPO in transfected cells after exposure to Tat-PNA 1 suggested that, in addition to penetration, an inhibitory antisense effect had been accomplished.[19,32,33] Thus, hybridization with the mRNA should have taken place. This result was further confirmed statistically with the radioactive labeled Tat-PNA 1 (see subsequently).

In addition to penetration and hybridization, labeled PNAs must be able to leave cells and tissues.[14,34] To study this aspect, a radioactive labeled construct (^{123}iodine-labeled Tat-PNA 1), more similar to the one than can be developed for in vivo image detectability, was also investigated. In this case, results were quantitatively compared with the penetration in nontransfected cells, showing a clear preference for accumulation of radioactivity in EPO-expressing cells in higher amounts than in wild-type cells. By exposing the cells that had incorporated Tat-PNA 1 to fresh media, a large part of the radioactivity present in both transfected and nontransfected cells was able to leave those cells, only a part remaining inside the cells for several hours. Radioactivity inside the transfected cells remained longer and higher than in nontranfected cells, probably as a result of the hybridization with mRNA. In fact, even the small absolute amount of ^{123}iodine-Tat-PNA persistent in those cells was also able to inhibit[35,36] part of the expression of EPO, as depicted in Figure 2C.

Thus, ^{123}iodine-Tat-PNA 1 was able to enter cells with higher amounts penetrating transfected cells than wild-type cells and was also able to leave such cells at a slow rate. Both accumulation and persistence was higher in transfected cells than in nontransfected cells. So, labeled-Tat-PNA 1 appears able to differentially image in vitro the presence of cells producing *Epo* mRNA from nonproducing cells by temporarily accumulating more radioactivity in the latter.

A pilot experiment in living animals appeared important to verify the possibilities of the approach for in vivo imaging.[6] This preliminary in vivo study was carried out with mice to which *Epo* gene transfer had been performed on their right leg by electroporation 15 days before. Both the increase of EPO in serum and the evolution of the hematocrit indicated the successful gene transfer process.[37] Two transferred mice and one nontransferred mouse were subjected to systemic[38] ^{123}I-Tat-PNA application. The results indicated a preferential uptake of radioactivity (especially for ^{123}I-labeled Tat-PNA 1) by the limb in which gene transfer had been carried out. The preferential uptake was revealed by a semiquantitative

estimation of the difference in radioactivity present in both legs, indicating that Tat-PNA 1 (and Tat-PNA 2 to a lower extent) presented a preferential uptake in the right (transferred) limb as compared with the non transferred control animal. Considering the pharmacokinetics of serum EPO (Fig. 3A), a greater effect could be expected when the time of the labeled Tat-PNA application overlaps with maximum EPO expression.[39] Thus, the slight preferential accumulation observed points to future in vivo experiments designed to obtain optimum detection capability.

In summary, cell penetrability of PNAs when coupled covalently to appropriate peptide carrier has been verified by means of fluorescence and radioactive measurements. A partial antisense inhibitory effect on EPO expression has been detected, confirming PNA penetration and hybridization with mRNA. Subsequently, Tat-PNA leaves the cells steadily, but a substantial amount of it remains in transfected cells for several hours in a higher proportion than in control, nontransfected cells. A pilot in vivo experiment in gene-transferred mice indicated that some differential detectability is possible also in vivo between the gene-transferred limb and the nongene-transferred limb. A further refinement of methodologies is expected to result in clear differences to be observed easily through external imaging techniques, especially incorporating higher sensitivity and resolution by means of positron emission tomography or single photon emission computerized tomography labeled Tat-PNA analogs. The prospect of external diagnostics of successful gene therapy applications appears to be promising by improving the basic methodology presented here.

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