ARTICLE
Comparative analysis of lentiviral vectors and modular protein nanovectors for traumatic brain injury gene therapy

Maria Luciana Negro-Demonte1,2, Paolo Saccardo3–5, Cecilia Giacomini2, Rafael Joaquín Yáñez-Muñoz2, Neus Ferrer-Miralles3–5, Esther Vazquez1–3, Antonio Villaverde3–5 and Hugo Peluffo1,2

Traumatic brain injury (TBI) remains as one of the leading causes of mortality and morbidity worldwide and there are no effective treatments currently available. Gene therapy applications have emerged as important alternatives for the treatment of diverse nervous system injuries. New strategies are evolving with the notion that each particular pathological condition may require a specific vector. Moreover, the lack of detailed comparative studies between different vectors under similar conditions hampers the selection of an ideal vector for a given pathological condition. The potential use of lentiviral vectors versus several modular protein-based nanovectors was compared using a controlled cortical impact model of TBI under the same gene therapy conditions. We show that variables such as protein/DNA ratio, incubation volume, and presence of serum or chloroquine in the transfection medium impact on both nanovector formation and transfection efficiency in vitro. While lentiviral vectors showed GFP protein 1 day after TBI and increased expression at 14 days, nanovectors showed stable and lower GFP transgene expression from 1 to 14 days. No toxicity after TBI by any of the vectors was observed as determined by resulting levels of IL-1β or using neurological sticky tape test. In fact, both vector types induced functional improvement per se.

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INTRODUCTION
Traumatic brain injury (TBI) is a leading cause of mortality and morbidity in both industrialized and developing countries, being of greater importance in poorer countries.1,2 TBI is frequently referred to as the “silent epidemic” because beyond symptoms such as paralysis, additional complications affecting intellectual ability, sensation, language, and emotion may not be readily apparent. In fact, studies that included several European countries showed that TBI resulted in one of the highest injury burden pathologies due to permanent disability,3 and among the highest costs for health systems.4 Extensive efforts have been made to develop neuroprotective therapies for this devastating disorder, but despite interesting preclinical results, no successful outcomes have been observed in human clinical trials to date.1,2 Following an initial mechanical insult, focal TBI results in complex delayed secondary progressive injury due to anatomical, neurochemical, metabolic, inflammatory, and cellular changes that account for many of the neurological deficits observed.1,2,5

The introduction of functional genes into an organism for treating chronic disorders of the central nervous system (CNS),6,9 One main focus in gene therapy research is the development of appropriate delivery systems,6,7,10 which can be complicated and is considered to be a bottleneck for achieving desired clinical outcomes. Viral vectors tend to be the most effective carriers of nucleic acids into foreign cells, as they are evolutionary optimized for this purpose. One of the most popular types of viral vectors being developed for treating CNS disorders are HIV 1-derived lentiviral vectors.11,12 They have been tailored in recent years to have reduced biological risk and to have features that make them excellent delivery vehicles including applications for treating CNS disorders. These vectors are safe, have low immunogenicity and transduce postmitotic cells.13–15 Nonintegrating lentiviral vectors, in particular, show promising features for use in treating CNS disorders.16,17 Nonviral vectors have also gained attention,6,7,10,18 and in particular, vehicles based on multifunctional proteins in DNA complexes. These vectors constitute a very versatile type of carrier for therapeutic nucleic acids, constructed by combining appropriate functional domains fused as a single polypeptide chain.19 This approach has generated the first prototypes of modular recombinant protein nanovectors where the integrated domains enable the construct to mimic an infective viral cycle. In this way, delivery of nucleic acids is effectively targeted. This type of nanoparticle has also been termed “artificial
The modular nature of such constructs allows for selection and incorporation of well-characterized peptides with different features so as to improve function by iteratively redesigning the construct. Several nanovectors of this type can be successfully used to transfect cells in vitro and to result in the expected therapeutic effect when used in vivo. There is thus great interest in developing the potential of these nanovectors for use in a clinical context. One such modular nanovector, i.e., NLSc, is based on a tetrameric carrier protein including β-Galactosidase engineered with a polylysine K10 tail which binds to and condenses DNA, a NLS motif for nuclear localization and an prototypic integrin-interacting RGD domain, which binds to membrane integrins to promote cell internalization. This vector was shown to induce biologically relevant concentrations of transgenic protein after acute excitotoxic brain injury. The NLSc protein RGD interacting motif could induce neuroprotection per se raising the interesting possibility of utilizing vector functional motif activities to rapidly direct neuroprotective actions on the specific target. The modular principles underlying the NLSc vector were further improved by generating two smaller nanovectors termed HKRN and HNRK. These polypeptides are based on alternative combinations in a single small polypeptide of domains RGD, NLS, and K10 and an additional poly-histidine domain H3 that provides for endosomal escape and is also useful for purification of these constructs. These nanovector constructions could be used to achieve significant levels of transgene expression in cultured cells, but testing in vivo transfection properties have not been done.

Importantly, the original hypothesis that a single very efficient gene therapy vector could be used for most applications has been revised to the idea that each particular pathological condition may require a specific vector. For instance, for treatment of acute traumatic CNS injuries, the vector should induce a rapid but nonpermanent induction of transgene expression, it should not be proinflammatory as inflammation is a key mediator of neuropathology and a specific cell type may or may not needed to be targeted depending on the mechanism of action of the transgene. In addition, other less obvious variables have become important to consider: (i) which are the optimal levels of transgene expression? (ii) What is the ideal time window for expression? (iii) Should the vector induce widespread or a localized transduction? Moreover, the lack of detailed comparisons of different vector types in the same model under identical conditions continue to hamper selection of the most appropriate vector for particular pathological conditions.

In this context, the aim of the present study was to quantify, under similar conditions, the potential of lentiviral vectors versus the modular recombinant nanovectors NLSc, HNRK, and HKRN as gene therapy vectors for TBI. We first describe the effect of several variables such as protein/DNA mass ratio, incubation volume, and presence of serum or chloroquine in the transfection medium on nanovector formation and transfection efficiency in vitro. Moreover, using the controlled cortical impact (CCI) model for TBI, we evaluated the level and time-window of transgene expression, induction of inflammation, and toxic effects induced by both types of vector.

RESULTS

Modular protein nanovectors HNRK and HKRN were incubated with plasmid DNA at room temperature, to generate self-organized nanoparticles of ca. 80 nm. Since different protein/DNA mass ratios can result in different transfection efficiencies, we analyzed transfection efficiency of HNRK and HKRN vectors formed at different protein/DNA mass ratios. This was done taking into account that protein/DNA mass ratios of 2.5 and 1.5 of HNRK and HKRN, respectively, result in complete retardation of total DNA in the sample in gel retardation assays. HEK293T cells were incubated with nanovectors loaded with 2 μg of a GFP-expressing plasmid transcriptionally controlled by a CMV promoter. Percentage of cells transfected and the relative fluorescence intensity/cell were analyzed 24 hours after transfection. The HNRK and HKRN vectors purified from bacterial inclusion bodies had increased transfection efficiency when compared to the same vectors purified from soluble bacterial fractions (HNRKs), or when compared to the similar vector NLSc or to naked DNA (Figure 1a). Increased protein/DNA mass ratios in nanocomplexes formed resulted in increased transfection efficiency with optimal ratios of 4.5–5 (Figure 1a), and declining efficiency at higher ratios thereafter (not shown). Interestingly, although a higher percentage of cells were transfected with lipofectamine than with HNRK or HKRN nanocomplexes, relative fluorescence intensity/cell was similar for the three groups at 4.5–5 protein/DNA mass ratios (Figure 1b). Also, there was no decrease in cell viability after treatment with nanocomplexes (not shown). We next transduced HEK293T cells with third-generation lentiviral vectors pseudotyped with VSV envelope protein. These vectors carried the same expression cassette used in the nanovector transfection assay, and were analyzed 1 and 3 days post-transfection/transduction (dpt) for percentage of cells transduced and for relative fluorescence intensity/cell. Transfection of cells with HNRK or HKRN resulted in maximal transgene expression at 24 hours (not shown), however and as previously reported lentiviral vectors required at least 3 days for full transgene expression (Figure 1c, d). Surprisingly, GFP was detected as early as 1 dpt for all multiplicities of infection (MOIs) tested. Cell toxicity was observed at 3 dpt for the highest MOI tested (i.e., MOI = 4.4, data not shown). HEK293T cell transduction efficiency, however, was higher for lentiviral vectors (at MOI 1, there are 1.4 × 10^5 to 1.4 × 10^6 copies of the gfp gene in transfected cells according to QPCR data derived calculations in ref. 33) compared with the nanovectors (the 2 μg DNA used per well corresponds to 2.4 × 10^1 copies of gfp gene) in terms of percentage of transfected cells (Figure 1a–d). In terms of relative fluorescence intensity/cell, qualitative microscopic observations confirmed the increased intensity after lentiviral transduction when compared to nanovector transfection. Comparative quantitative data are shown in Table 1.

Transfection efficiency of nanovectors for applications in gene therapy relies on their ability to escape endosomes after being endocytosed. We show that transfection efficiency with both HNRK and HKRN can be increased, especially the HKRN vector, using the prototypic endosome destabilizing agent chloroquine (Figure 2a, b). Serum proteins are another factors known to affect transfection efficiency for different vectors. We show that the presence of 10% serum during transfection of HEK293T cells results in an ~40 and 35% decrease in the transfection efficiency for HNRK and HKRN, respectively (Figure 2c, d).

Another limiting factor for in vivo administration of these vectors is the volume that can be injected into CNS parenchyma due to very limited extracellular space. Thus, we evaluated the effect of nanocomplex formation in a reduced volume. A reduction in nanocomplex formation volume, i.e., from 200 to 30 μl OPTIPRO medium, however, resulted in 52 and 42% decreases in HEK293T cell transfection efficiency for HNRK and HKRN nanovectors, respectively (Figure 3a, b). Another important variable that can affect nanocomplex formation and transfection efficiency is protein/DNA incubation time. Analysis of HNRK/DNA nanocomplex particles formed using dynamic light scattering (DLS) and transmission electron microscopy (TEM) indicated particle size tended to be less variable when produced in 200 μl OPTIPRO medium compared with being
produced in 30 μl phosphate-buffered saline (PBS), the vehicle used for in vivo administration of vectors (Figure 3c,d). There were no significant differences in nanocomplex formation with either 20 minutes or 5 hours incubation times for either condition (200 μl OPTIPRO or 30 μl PBS). Accordingly, no differences were observed in HEK293T cell transfection efficiency of HNRK/pDNA nanovectors.
formed in 200 μl OPTIPRO over 20 minutes versus those formed over 5 hours (Figure 3e). Nanocomplex size observed by TEM and hydrodynamic diameter observed by DLS differed. While TEM images showed particles to be between 20–200 nm, DLS indicated particles to be between 1–2 μm suggesting clustering or aggregation of particles.

Selection of an appropriate vector for a given gene therapy application is critical. In order to determine which vector is most suited for treatment of TBI, we compared several important variables of HNRK/pDNA nanovectors and third-generation lentiviral vectors under identical conditions using a CCI TBI injury model. The previously described nanovector, NLSCt/pDNA, was also included as an internal standard. This vector was shown to be able to induce transgene expression after acute excitotoxic brain injury, and to result in producing significant biological effects. Comparisons of relative levels of GFP protein after CCI lesion were made by enzyme-linked immunosorbent assay (ELISA) of samples from untreated control rats and those receiving a 4 hours delayed intraparenchymal injection of nanovectors NLSCt/pDNA or HNRK/pDNA or lentiviral vectors. GFP protein was detected in all three cases at 1 dpl postlesion (dpl) (Figure 4a). The GFP level at 1 dpl was 100-fold increase in GFP protein levels comparing 1 and 14 dpl after lentiviral injection tended to increase compared with both NLSCt/pDNA- and HNRK/pDNA-treated animals. There was a 100-fold increase in GFP protein levels comparing 1 and 14 dpl after lentiviral injection. Interestingly, animals subjected to CCI spontaneously recovered from the neurological deficits measured by this test over time, hinting further evaluation after 14 dpl (not shown). A very similar profile was observed after lentivector injection, showing less pronounced functional compromise at 14 dpl (Figure 4d).

This vector was shown to be able to induce transgene expression after acute excitotoxic brain injury, and to result in producing significant biological effects. Comparisons of relative levels of GFP protein after CCI lesion were made by enzyme-linked immunosorbent assay (ELISA) of samples from untreated control rats and those receiving a 4 hours delayed intraparenchymal injection of nanovectors NLSCt/pDNA or HNRK/pDNA or lentiviral vectors. GFP protein was detected in all three cases at 1 dpl postlesion (dpl) (Figure 4a). The GFP level at 1 dpl was 100-fold increase in GFP protein levels comparing 1 and 14 dpl after lentiviral injection tended to increase compared with both NLSCt/pDNA- and HNRK/pDNA-treated animals. There was a 100-fold increase in GFP protein levels comparing 1 and 14 dpl after lentiviral injection. Interestingly, animals subjected to CCI spontaneously recovered from the neurological deficits measured by this test over time, hinting further evaluation after 14 dpl (not shown). A very similar profile was observed after lentivector injection, showing less pronounced functional compromise at 14 dpl (Figure 4d).

DISCUSSION

Many gene therapy vectors induce inflammation, which may result in toxicity due to the treatment. It is very important to avoid these effects in treating TBI, where inflammatory reactions are key responses in development of subsequent neuropathology. Determination to what extent our vectors elicits inflammatory responses is thus critical for their development. We analyzed levels of IL-1β, the prototypic proinflammatory marker, after CCI and injection of the different vectors. No significant differences were observed comparing vehicle-injected and nanovector-injected animals (Figure 4b). To further evaluate possible toxic effects of vectors, the sticky tape functional test was performed to evaluate motor and sensory function (Figure 4c,d). As previously described, when this test was done with both nonlesioned animals or on the ipsilateral forepaw of lesioned animals, a very rapid (2–5 seconds) detection and removal of the tape results (not shown). After CCI, animals show increased time for detection and removal of the sticky tape placed on the contralateral forepaw. In accordance with studies on neuroprotection after excitotoxic brain lesions, NLSCt/pDNA-treated animals in fact displayed less pronounced functional compromise at 10 days after CCI (Figure 4c). Administration of the nanovector HNRK/pDNA also resulted in less pronounced functional compromise at 10 dpl. Interestingly, animals subjected to CCI spontaneously recovered from the neurological deficits measured by this test over time, hinting further evaluation after 14 dpl (not shown). A very similar profile was observed after lentivector injection, showing less pronounced functional compromise at 14 dpl (Figure 4d).
Figure 3  Effect of incubation volume and medium on the protein nanovector complex size and transfection efficiency. HNRK and HKRN modular protein nanovectors were allowed to self-assembly during a 20-minute incubation of HNRK and HKRN proteins with plasmid DNA in either 200 μl OPTIPRO medium or 30 μl PBS. Assembled nanovectors were then used to transfect HEK293T cells and transfection efficiencies were quantified 1 day later by flow cytometry (a, b). HNRK nanovector stability was analyzed after self-assembly in 200 μl OPTIPRO medium or 30 μl phosphate-buffered saline, and for 20 minutes to 5 hours incubation periods. The products formed were analyzed by dynamic light scattering (c) or transmission electron microscopy (d) as indicated. Transfection efficiency of HNRK nanovector after self-assembling in 200 μl OPTIPRO medium for 20 minutes to 5 hours was analyzed by flow cytometry 1 day after transfection (100% represents transfection efficiency for nanovectors formed in the 20 minutes incubation time). *P < 0.05 when compared to vectors allowed to self-assembly in 200 μl OPTIPRO.
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and for at least 2 weeks. Transgene expression levels were much higher and more variable using the lentiviral vectors, and no apparent increase in inflammation or neurodegeneration was observed after treatment with any of the vectors. In fact, the injection of either vector type per se induced functional recovery of the animals.

There remain several obstacles that need to be better understood for the development of such vectors for use in therapeutic applications and other in vivo conditions. Direct injection of vectors into the brain parenchyma limits their delivery to a relatively small volume of tissue due to the very limited CNS extracellular space. Direct intraparenchymal injection of vectors into the human brain by stereotactic methods, as in Parkinson’s disease clinical trials, on the other hand can be accomplished without important permanent side effects or intracerebral hemorrhages, and also have resulted interesting clinical outcomes. In the human brain, up to 80 μl can be injected at a given site. No more that 5 μl, however, is normally injected per injection site in rat brain. To increase amount and distribution of vector administered in our study, three injections of 2 μl each were administered in the anterior, lateral, and posterior lesion penumbra regions. In an attempt to further concentrate vector administered for a given injection, we conducted our standard NHRK nanovector self-assembly normally done in a 200 μl volume in a smaller volume of 30 μl. Self-assembly of vectors into nanoparticles by combining modular protein with pDNA is a very interesting and useful phenomenon enabling assembled vectors to act as efficient vehicles for nucleic acid transport to the cell nucleus.

Figure 4 Comparative efficiency and toxicity of protein nanovectors and lentiviral vectors after a controlled cortical impact (CCI). Four hours after a CCI, animals were intracortically injected with either protein nanovectors or lentiviral vectors at three locations in the lesion border (see Materials and Methods). At 1 and 14 days dpl, animals were sacrificed and levels of GFP protein analyzed by enzyme-linked immunosorbent assay (ELISA) (a, *P < 0.05 when compared to all other groups). The effect of vectors on proinflammatory responses after CCI treatment was analyzed by measurement of IL-1β expression levels by ELISA and compared with vehicle-treated CCI animals (b), and no significant differences were observed. The putative toxicity of the vectors after a CCI was evaluated by the sticky tape functional test (c, d). The mean time elapsed for removal of the sticky tape on the lesioned forepaw is shown for each group from 3 to 14dpl. Phosphate-bufffered saline (PBS) n = 12, Dulbecco’s modified Eagle medium (DMEM) n = 8, NLSCT n = 7, HNRK n = 13, lentivector n = 9. *P < 0.05 when compared to PBS at 10 dpl (c); *P < 0.05 when compared to DMEM at 14 dpl (d).
Furthermore, and consistent with other studies,10,24 we found an optimal protein/pDNA mass ratio for nanovector self-assembly and transfection efficiency. The optimal protein/pDNA mass ratio for assembly and transfection efficiency was ca. 5 for both HNRK and HKRN protein nanovectors. There are 2,284 HNRK or HKRN proteins/plasmid molecule, resulting in a predicted 7,529 net positive charges/nanovector molecule at physiological pH. Kenny and colleagues have shown that a similar anionic Neurotensin receptor targeted nanovector showed increased diffusion in noninjured brain parenchyma when compared to its cationic counterpart.41 This suggests the interesting possibility that a similar approach with our nanovector could increase its brain distribution. However, one has to consider if a widespread distribution is always desired, as under some conditions a specific nuclei or region of the brain may need to be targeted. Moreover, after acute brain injury significant edema occurs, potentiating diffusion of molecules in the entire lesioned area. Accordingly, diffusion of vector in the entire lesioned area has been shown for the 249AL vector, which is almost identical to the NLSc vector used here.42

HNRK/pDNA or HKRN/pDNA nanocomplex formation in 30 μl PBS rather than 200 μl OPTIPRO resulted in the formation of complexes with about 50% decreased transfection efficiency using the same conditions for transfection of cultured HEK293T cells. This decrease in transfection efficiency for particles formed in the smaller volume correlated with higher variability in particle size as determined by TEM or DLS. These changes in apparent particle size may affect transfection efficiency and may reflect differences in pDNA compaction, altered protein functional domain exposure, particle clustering or pDNA release from particles inside the cell. Increased particle size may also affect the diffusion in the lesioned brain. How variation in particle size may affect their transfection efficiency will require further study. Understanding processes and mechanisms of nanocomplex formation might allow for refining efforts to form more uniform particles in smaller volumes and that have improved transfection efficiency. Lentiviral particle preparations do not aggregate when added to cell cultures,43 however comparing possible aggregation after intraparenchymal injection should be analyzed.

HNRK and HKRN modular proteins display a cell-targeting domain (RGD), a DNA attaching/condensing domain (K tail), a nuclear localization domain (NLS) and a poly-histidine tail that can stimulate endosomal escape. However, endosomal escape may still need to be improved further to increase transfection efficiency. In this regard, we included endosome-disrupting agent, chloroquine, in the cultures to evaluate putative increases in transfection efficiency. Chloroquine addition indeed resulted in increased transfection efficiency for both HNRK and HKRN nanovectors, with more pronounced effects for the HNRK vector. This suggests engineering the HNRK vector can be further improved using other known endosome escaping motifs.13 Alternatively, chloroquine could be co-injected with assembled nanovector in vivo to increase transfection efficiency, as chloroquine has been approved for use in human subjects. Finally, instead of performing an intracerebral injection, protein nanovectors could be administered by an intravenous route and may reach the brain after TBI where the blood–brain barrier has been altered. Many vectors fail to induce significant transgene expression levels when combined with serum, including lentiviral vectors.24 Lentiviral vectors are inactivated by the serum complement system14 and vector activity decreased ~2 logs within 45 minutes after intravenous administration.48 This problem can be overcome using lentivectors pseudotyped with the VSV-G protein subjected to directed evolution,49 or by conjugating the VSV-G envelope protein with poly(ethylene) glycol.46 Nonviral vectors may also show decreased transfection efficiency in vivo due to serum interactions, and the addition of different shielding molecules, e.g., N-succinyl-chitosan/polyethylenimine can be included to contribute to enhanced stability in the presence of serum.46 Here, we show that the presence of 10% serum induced a 35–40% decrease in HEK293T cell transfection efficiency for both nanovectors. This suggests that intravenous administration may need further protection of the vectors as described above to be an effective way to induce efficient transgene expression in target brain cells. Unpublished results from our laboratory indicate that intravenous administration of the NLSc and the HNRK vector induced gfp transgene expression in several organs, but this issue needs further in vivo testing.

Only few reports have focused on gene therapy applications for acute CNS injuries like TBI. In some studies, expression of a protective transgene is induced prior to TBI. Minnicich et al.,47 e.g., overexpressed GDNF with adenoviral vectors (AdV) 1 week before CCI, and observed neuroprotection for up to 2 weeks.47 However, when GDNF AdV vectors were injected immediately after CCI, no neuroprotection was observed unless L-arginine was included in the injection administered.48 A comparative gene therapy study using a fluid percussion injury TBI model was recently reported by Siddiq et al.,49 were overexpression of a transactivator of vascular endothelial growth factor A using AdV and adeno-associated viral vectors was performed. Interestingly, the two vector types have different temporal transgene expression profiles. AdV vectors show high initial levels of transgene expression but that is attenuated at longer time point,46 while adeno-associated virus (AAV) exhibited slower but long-lasting transgene expression and as well as fewer proinflammatory responses.51 Siddiq et al. injected identical pfu/ml of either vector in 2 μl per injection site 20 minutes after the fluid percussion injury and observed that overexpression of VEGF-A using both vectors had beneficial effects, and included a 30-day functional improvement mediated by AAV. In this study, different parameters for AdV and AAV were analyzed in treated animals precluding a comprehensive comparison of the two vectors.

AdV expression of a transgene under the control of a CMV promoter in the CNS was shown to peak at 4–8 days after injection and decline by 14 days.50 This decline in expression was found to be a consequence of immunological responses directed against cells with transgenes.52 The use of CMV promoter driven integration proficient and deficient lentiviral vectors16 as well as CBA promoter driven AVV41 for CNS transduction were shown to induce persistent transgene expression in immunocompetent animals, and should thus be useful as vectors for targeting CNS tissues. Here, we show that even after TBI, where inflammation is greatly potentiated by lesion progression, transgene expression did not decline for at least 14 days using any of the vectors. In fact, we also found that there was a significant increase in transgene expression mediated by lentiviral vectors at 14 dpl. There was also a trend to increased expression with use of the HNRK vector compared with use of the NLSc vector. An increase in transgene expression was also evident from 1 to 3 days in lentiviral transduced HEK293T cultured cells. Dorsal root ganglion neurons also showed both an increase in % GFP positive cells and in GFP fluorescence/cell from 2 to 8 days after transduction using the same lentiviral system.41 In our study, however, GFP protein was detected at 1 dpt both in vitro and in vivo. Transgene expression by lentiviral vectors has not been documented previously at 1 dpt, and then GFP protein observed after lentiviral transduction might be due to carry-over during vector production or pseudotransduction. Independently of the origin of GFP, its presence at 1 dpt may contribute to neuroprotection after TBI, were the therapeutic window is narrow and thus a rapid treatment is essential.
Variability in transgene expression levels despite using highly controlled procedures is a concern that remains difficult to resolve. However, data showing individual expression levels are normally not reported and thus further underscore the importance of this problem. We observe that lentivectors and to a lesser extent protein nanovectors can show significant variation in the levels of transgene expression. Each animal received three independent 2 μl injections using a Hamilton syringe and an automatic nanoinjector at 0.4 μl/minute and leaving the syringe in place for 5 additional minutes, making it highly unlikely that, e.g., blockage of the syringe or reduced diffusion would account for such variation in transgene expression. Another potential contribution to this variability could be the TBI procedure or the evolution of delayed secondary progressive injury. However, injection of either integrative proficient or integrative deficient lentiviral vectors into nonlesioned spinal cord of mice and rats also resulted in some animals having high levels of transgene expression and others with very low levels of expression. This was seen despite use of a carefully controlled injection procedure and use of identical vector batches, as in the present study. Individual variations in immune responses against the transgene or the vectors, or fluctuation of injections near or farther away from blood vessels may account for differences in GFP expression levels observed between animals. Taken together, this indicates that variability may be due to unknown technical issues or to the vectors, but not to TBI-related variables. Other methods like convective enhanced delivery associated with different catheters used in clinical trials delivering nanoparticles or viral vectors may provide the means for more reproducible delivery of vectors. In addition, no correlation was found between self-assembly reaction times for protein and pDNA and resulting GFP transgene expression in vivo (not shown). Also, no significant variation in transfection efficiency for HEK293T cells was found for either 20 minutes or 5 hours self-assembly reaction times, suggesting that this variable does not account for the in vivo transgene level expression variations. Further studies need to be performed to establish to what extent variations in in vivo transgene expression levels are due to experimental conditions in the overall gene therapy procedure in small animals or associated to other variables such as immune response.

The time frame for TBI treatment is short in most neuroprotective strategies, and thus using a gene therapy approach should involve vectors that rapidly express a transgene. In the fluid percussion injury TBI model mentioned above, where vectors were administered 20 minutes after by intraparenchymal injection, AdV and AVV were shown to induce transgene expression at 3 days (first time studied) and not before 14 days, respectively. There are no other studies in which transgene expression response time has been analyzed at shorter times after acute brain injury. In this study, we show that both NLSc and HNRK nanovectors and lentiviral vectors when administered 4 hours after CCI result in significant GFP protein levels at 24 hours after CCI. The therapeutic strategy used in this study includes a 4-hour interval between CCI and treatment which approximates the time it make take from time of TBI cranial injury to that of diagnosis and possible treatment in a well-organized clinical context. Thus, rapid GFP transgene expression observed with HNRK nanovector and the presence of GFP protein after lentiviral vector injection induced by carry-over or pseudotransduction suggests that they could provide an appropriate expression profile for treatment of at least the initial phases of TBI. Further comparative studies are needed to determine if these vectors can effectively provide neuroprotection after a CCI. Interestingly, nanovector NLSc was reported to induce neuroprotection under different acute excitotoxic conditions and with different transgenes when injected intracortically 4 hours after lesion generation. Moreover, the expression of toxic transgenes with the same vector increased excitotoxic lesion. We report here that the HNRK vector tends to produce a stable level of transgenic protein until 14 dpl, while the lentivector induced a significant increase in the levels of transgenic protein up to 14 dpl. The HNRK vector could be re-injected to prolong the transgene expression over time, but a careful evaluation of the putative adaptive immune response elicited toward the vector itself should be evaluated. Taken together, these results suggest that both nanovectors and lentiviral vectors can result in expression of biological relevant concentrations of transgenic protein in vivo after a CCI, and provides flexibility in selection of the most appropriate vector for neuroprotection after TBI.

Neuroinflammatory cascades are major contributors to secondary progressing injury, and thus additional inflammatory reactions promoted by gene therapy vectors may constitute a problem. We show here that no increase in levels of prototypical proinflammatory cytokine IL-1β was observed after injection of any of the vectors, suggesting that no further inflammation is produced. Moreover, no toxic effect for any of the vectors used were noted by the functional sticky tape test. In addition, both NLSc and HNRK nanovectors exhibited functional neuroprotective effects after CCI. The NLSc vector alone (not harboring pDNA) was also previously shown to be neuroprotective after acute excitotoxic brain injury. The mechanism involved its RGD integrin-interacting motif and interaction with glial cells. In addition, the HNRK and HKRN vectors without pDNA were also shown to be neuroprotective for cultured PC12 cells. Thus, data presented here confirm the neuroprotective potential of these nanovectors per se in a clinically relevant TBI model. Surprisingly, the lentiviral vectors preparation also showed a significant functional neuroprotection using the sticky tape test, and the beneficial effects of these vector preparations without expressed transgenes should be studied in detail.

The lack of extensive comparative studies using several vectors under the same experimental TBI conditions hampers the selection of putative ideal vectors for clinical applications. Our study aimed to contribute to this important clinical need by comparing two of the most promising prototypes, i.e., modular nanovectors and lentiviral vectors. Our data indicates that both the HNRK protein nanovector and the lentiviral vectors are very useful prototypes for gene therapy applications for treatment of TBI due to the rapid and non-toxic and non-proinflammatory expression of a transgene. As already stated, this is not the case for AAV which need at lest 14 dpt to show the presence of the transgene. However, they observed a beneficial functional improvement at 30 dpt by overexpressing VEGF-A, suggesting that the selection of the ideal vector for the treatment of TBI must be considered in the context of the neuroprotective transgene of choice and its timing of expression for effective neuroprotection. HNRK nanovector showed rapid and prolonged stable expression while lentiviral vectors show slower and increasing levels of expression with time. As inferred from the limited data available for AAV in TBI models, they show a similar profile of time-course transgene expression as lentiviral vectors, with variable expression levels at different time points. The regulation of expression of the transgene is critical for adjusting the dosage for treatment. This could potentially be achieved using inducible promoters for the expression from lentiviral vectors and AAV as reported. Dosage of transgene expression driven by modular nanovectors would be more simple. Adenoviral vectors induce significant inflammatory reactions leading to the rapid elimination of the transgene expression in the brain. For this reason and due to the fact that the inflammatory cascades are an important part...
of the physiopathology of TBI, these vectors may not be adequate for the treatment of TBI. Neither of the vectors tested here contributed strongly to the neuroinflammation provoked by CCI, a profile probably shared by AAV, suggesting that they are all suitable for the treatment of TBI. Vectors can also be expected to differ in terms of cell tropism, which again should be carefully considered in context of the mechanism of action of the selected transgene. All three vectors have a broad tropism that can in addition be modulated by surface protein domains, and are for thus useful vectors. Some AAV serotypes have been shown cross the blood brain barrier, and thus have good potential for TBI treatment by intravenous administration. 57 On the other hand, local intraparenchymal injection of the vectors for TBI treatment may be a wiser choice to avoid intravenous injection of high titers of vectors that may have a negative impact. Taken together, one or more of these vectors may prove to be suitable for the treatment of TBI, but its choice should consider the specific neuroprotective mechanism of the transgene used. Importantly, critical properties for development of vectors as clinical formulations have to be taken into account. The ability of these nanovectors to readily self-assemble at room temperature self-assembly with no effect of incubation times on nanovectors transfection efficiency is an important step toward this goal. Also these vectors are noninfective, and the protein can be lyophilized with no loss of activity. In this regard, these vectors would be of better choice over the viral vectors if the mechanism of action of the neuroprotective transgene involves rapid, short-term and low expression levels. However, if a long-term and high-level expression is needed to achieve neuroprotection, both non-integrated lentiviral and AVV may prove to be more suitable. Finally, the functional improvement observed by the modular nanovector and the lentiviral vector preparations per se opens the way for the design of additional combinatorial tools for neuroprotection.

MATERIALS AND METHODS

Nanovektor production and purification
The production of both chimerical proteins HKRN and HNRK, cloned in plasmid pET-28a (+) (TEV), was induced with addition of 1 mmol/l Isopropl β-D-1-thiogalactopyranoside (IPTG) to plasmid-containing BL21 (DE3) Escherichia coli cell cultures (at OD600 nm = 0.4–0.6) growing in Luria–Bertani medium (Sigma-Aldrich, Chemie GmbH Munich, Germany) at 37 °C. After 4 hours, cells were harvested by centrifugation, washed with PBS, and stored at −80 °C. The bacterial pellet was suspended in lysis buffer 20 mmol/l Tris-HCl pH 8.0, 0.5% mmol/l NaCl, 6 mol/l guanidinium chloride, and cells were disrupted by sonication in the presence of ethylenediamine tetra acetic acid (EDTA)-free protease inhibitor cocktail tablets (Sigmafast Cat. No. 38820, Sigma-Aldrich). The soluble fraction was obtained by centrifugation at 15,000 g for 45 minutes at 4 °C and further purified using 0.22 μm filters. Protein-containing fractions were pooled and passed through a PD-10 desalting column (GE Healthcare) using 0.05 mol/l Tris-Cl buffer pH 7.5, and cells were disrupted by sonication in the presence of EDTA-free protease inhibitor cocktail tablets. Transmission electron microscopy

Transmission electron microscopy

The pRRLs in_PPT_CMV_GFP pre endotoxin-free vector described above and having the GFP marker to monitor transfection efficiency, was also used for construction of nanovektors. HKRN/pDNA and HNRK/pDNA nano complex self-assembly was done using individual 5 minutes incubations with different amounts of protein in 100 μl OPTIPRO medium (Gibco Life Technologies, Grandisland, NY) and 2 μg plasmid DNA in 100 μl OPTIPRO medium. Protein/DNA complexes were generated by mixing 100 μl DNA and 100 μl protein at specified ratios, and incubating at room temperature for 20 minutes or indicated times. The 200 μl mixture was then administrated to HeK293T/17 cells (Invitrogen, Carlsbad, CA) in 24-well plates grown to 70–80% cell confluence for 4 hours in the absence of serum. Subsequently, the medium was changed to include serum and cells were incubated for an additional 20 hours. Choroiquin 200 mmol/l (C6628, Sigma Aldrich) or 10% of fetal bovine serum (Gibco Life Technologies) were added to the cells at the moment of incubation with the protein/pDNA nanocomplexes to test the ability of complexes to escape endosomes and to transfect cells in the presence of serum respectively. Transfection efficiency of nanocomplexes formed in smaller volumes was also tested. Complex self-assembly in these cases was done with identical ratios and quantities of protein and pDNA.
as used in the 200 μl final volume experiments above but in 30 μl PBS. The transfection efficiency was quantified 24 hours after transfection using GFP reporter gene expression analyzed by flow cytometry in the FACS Calibur system using CellQuest software (BD Biosciences, Franklin Lakes, NJ). Results obtained when samples were visualized by fluorescence microscopy were consistent with flow cytometry gene expression analysis and negative controls (untreated cells and cells with plasmid only).

Animals, controlled cortical impact model, and vector injections All experimental work was approved by Comisión Nacional de Experimentación Animal and the CHEA-UDELAR ethical commission, and conducted according to directives of the Federation of Laboratory Animal Science Associations. Adult male Wistar rats (280–300 g) were submitted to CCI traumatic lesions under 3% isoflurane anesthesia and O2 (Abbott, Abbott Park, IL) as described previously. Briefly, rats were mounted in an injury device stereotaxic frame in prone position and secured by ear and incisor bars. The head was held in horizontal plane, and a midline incision was made aseptically in which soft tissues were reflected, and a 4 mm round craniotomy was made over the right primary motor and somatosensory cortex (coordinates to the center of the craniotomy: AP: Bregma, L: –1.7 mm). A stereotaxic mounted cortical impactor (PrecisionCortical ImpactorModel PCD3000 Hatteras Instruments, Cary, NC) was used with a 3-mm diameter tip (velocity: 1.5 m/s, contusion depth: 4-mm, contusion time: 120ms). Four hours later, 6 μl of vehicle (PBS or DMEM/10 mmol/l MgCl2), lentivirus (titer: 3.12 × 107 TU/ml, in DMEM/10 mmol/l MgCl2), and protein vectors accommodating an expression plasmid assembled in PBS (NLScSt protein/DNA ratio 33.3, 0.14 μg DNA per animal, or HNRK ratio 5 both, 1.44 μg DNA per animal) were injected using a stereotactic mounted nanoinjector (Quintessential Instruments). No antibiotics were used for all ELISA measurements.

Data processing and statistical analysis All results are expressed as mean ± standard error of the mean. Student’s t-test was used to determine significant differences (P < 0.05) between two experimental groups after determination of the normal distribution of the samples and variance analysis. Two-way analysis of variance followed by Bonferroni’s multiple comparison test was used to determine significant differences (P < 0.05) for experimental settings with more than two experimental groups or time points.

CONFLICT OF INTEREST The authors declare no conflict of interest.

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