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13. ABSTRACT (Maximum 200 Words)
In PD the insult to the dopamine (DA) neuron is posited to involve oxidative injury mediated by mitochondrial respiratory abnormalities and through participation of oxidative adducts made onto DA and presynaptic target proteins such as a-synuclein. The misfolding of a-synuclein engendered by oxidative adduct formation is hypothesized to be a critical participating process in Lewy Body formation and dopamine neuron compromise and death. Our central hypothesis purports that protein aggregates forming within dopaminergic neurons are seeded and require misfolded a-synuclein and that these aggregates are cytotoxic thereby contributing directly to neuron death. Thus targeting a-synuclein protein misfolding will enable the development of effective therapy. The main goal of this application is to identify and characterize humanized single chain antibodies (scFvs) that recognize structural epitopes on a-synuclein misfolding. Thus far, we have expressed and purified wildtype and a-synuclein; generated altered conformers of these proteins; screened for and identified synuclein-specific scFvs. Finally, we have begun the characterization of these synuclein-specific scFvs.

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Table of Contents

INTRODUCTION

Parkinson's disease (PD) is clinical syndrome triggered by disparate mechanisms. The clinical and neuropathological features are indistinguishable among the mechanistically different forms thus suggesting a convergent shared pathway. The disease invariantly produces loss of dopamine neurons in the substantia nigra (SN). The selective insult to the dopamine (DA) neuron is posited to involve oxidative injury mediated, in part, by mitochondrial respiratory abnormalities and through participation of oxidative adducts made onto DA and presynaptic target proteins such as α-synuclein. Convergence in PD pathogenesis has been greatly aided by the linkage between mitochondrial toxicant (e.g., MPTP, paraquat, rotenone) injury, inherited defects in turnover of the presynaptic and Lewy Body constituent protein α-synuclein, and involvement of cytosolic DA itself. Recent data directly implicate DA-quinone in the modification of α-synuclein and its inducement of misfolding (β-sheet conformation) and aggregation. ***Taken together these data support our central hypothesis that protein aggregates forming within dopaminergic neurons are seeded and require misfolded α-synuclein and that these aggregates are cytotoxic thereby contributing directly to neuron death.*** *We propose that either interference with aggregate formation or dissolution of existing aggregates will attenuate the pathophysiology of PD.* To this end, we employ an approach to rapidly identify humanized single chain antibodies (scFvs) that recognize structural epitopes on candidate pathogenic proteins. These scFvs bind pathogenic structural conformers with submicromolar affinity, can be engineered for secretion or cytosolic localization (intrabodies), and configured for high-level gene expression from viral vectors developed in my laboratory.

BODY

Our progress on this project is described below. We have divided it into 4 sections (I.A.-I.D.) to follow our original Specific Aims. In addition we have included the Statement of Work as it applies to Year 01.

STATEMENT OF WORK

In PD the insult to the dopamine (DA) neuron is posited to involve oxidative injury mediated by mitochondrial respiratory abnormalities and through participation of oxidative adducts made onto DA and presynaptic target proteins such as α-synuclein (α-syn). The misfolding of α-syn engendered by oxidative adduct formation is hypothesized to be a critical participating process in Lewy Body formation and dopamine neuron compromise and death. Thus targeting α-syn protein misfolding will enable the development of effective therapy. **The main goal of this application for Year 01 is to express, purify and alter the conformation of human α-syn and to identify humanized single chain antibodies (scFvs) that recognize this protein.** In years 02-05 we propose to identify the structural epitopes on α-syn that interact with the scFvs and utilize these scFvs to attenuate the pathology associated with α-syn misfolding.

PROGRESS

Specific Aim I: Identification of conformationally directed scFvs. Hypothesis: Altered structural forms of α-syn are needed to define conformer-specific ScFvs.

I.A. Expression of wildtype and mutant α-syn. *Rationale: Quantities of structurally homogeneous protein conformers will be needed for the screening of scFvs.*
Wildtype human α-syn as well as α-syn double and single mutants were expressed and purified from *E. coli* (BL21 DE3) following the protocol of Giasson et al. with minor modifications [1]. The yield of α-syn from one 500 ml bacterial culture is approximately 8 mg of purified protein. As shown in **Figure 1**, we have successfully expressed and purified all three forms of α-syn as demonstrated by silver stain and western blot analysis. Silver stain analysis of proteins subjected to denaturing polyacrylamide gel electrophoresis demonstrates a major band corresponding to the MW of α-syn. Furthermore, western blot analysis utilizing commercially available α-syn specific antibodies further demonstrates that the purified 16-19 kDa band is α-syn.

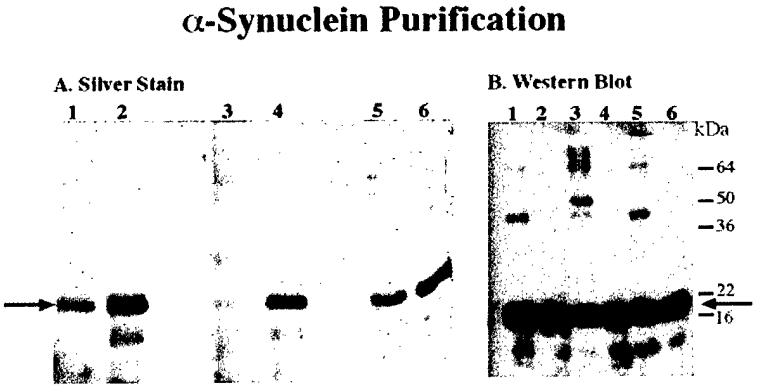

Figure 1. Demonstration of α-syn purity. Panel A. Silver stain analysis of total protein lysate before and after purification. Lanes 1, 3, & 5 represent induced bacterial cell lysates from cells transformed with WT, A53T & A30P α-syn, respectively (1 μg each). Lanes 2, 4, & 6 represent purified WT, A53T & A30P α-syn following biochemical purification (1 μg each). **Panel B. α-Syn western blot.** One μg of protein was subjected to polyacrylamide gel electrophoresis under denaturing conditions followed by western blot analysis with α-syn specific antibodies. Lanes are the same as in Panel A.

Regulated expression of wildtype and mutant α-syn in stable cell lines. A regulatable form of each α-syn type (wildtype, A53T, A30P) was prepared using a tetracycline-responsive autoregulated bi-directional expression vector [2]. Utilizing this system we have prepared stable cell lines expressing α-syn in a dose-dependent fashion. Regulated α-syn cell lines have been prepared in a mesencephalic-derived dopaminergic cell line (MN9D) for WT and A53T and in a cell line derived from mouse hippocampus (HN33.11) for WT, A53T and A30P. As shown in **Figure 2**, DOX-inducible α-syn expression increases in a time dependent manner.

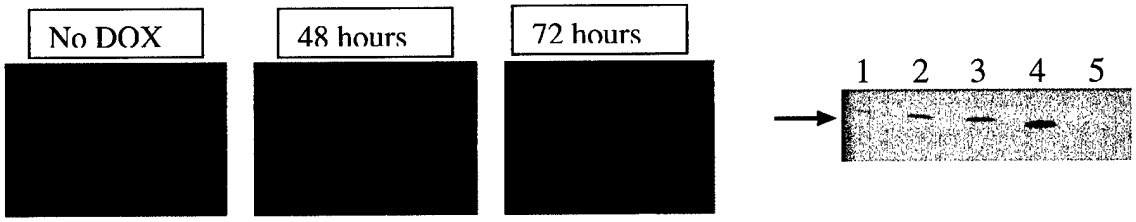

Figure 2. Regulated expression of α-syn in a stable cell line. MN9D cells were stably transfected with a doxycycline (DOX) regulated expression vector carrying the cDNA for A53T α-syn and an IRES green fluorescent protein cDNA. **Left Panel. α-Syn Immunocytochemistry.** Cells were treated with or without DOX for various times and subjected to immunocytochemistry utilizing antibodies specific to α-syn and fluorescent secondary antibodies. **Right Panel. α-Syn**

Western Blot Analysis. Cells were treated as described above, protein lysates prepared and 10 μg of lysate subjected to denaturing polyacrylamide gel electrophoresis followed by western blot analysis. Lanes 1-4 are derived from MN9D:A53Tα-syn cell lines without DOX treatment or with DOX for 48, 72 and 96 hours, respectively. Lane 5 is a protein lysate from the parental cell line (MN9D).

I.B. Generation of altered conformers. *Rationale: α-syn can exist in both the random coil (native) and β-sheet (misfolded) conformation (conformers). Random coil α-syn can be converted to the pathogenic β-sheet conformer and further pushed to aggregate formation by altering pH, temperature or oxidative adduct formation. On the other hand, mutant α-syn can exist as a β-sheet conformer in solution without these alterations.*
We have successfully produced altered conformations of WT, A53T and A30P α-syn. In one case the protein was incubated at 33°C, 1000 rpm for several days (shaking) and in the second case DA was added to the α-syn prior to and for the duration of the incubation (shaking+). Dopamine was included since it has been implicated as stabilizer of α-syn protofibrils [3]. As demonstrated by a Congo Red assay, α-syn aggregation increases following dopamine treatment (**Figure 3A**).

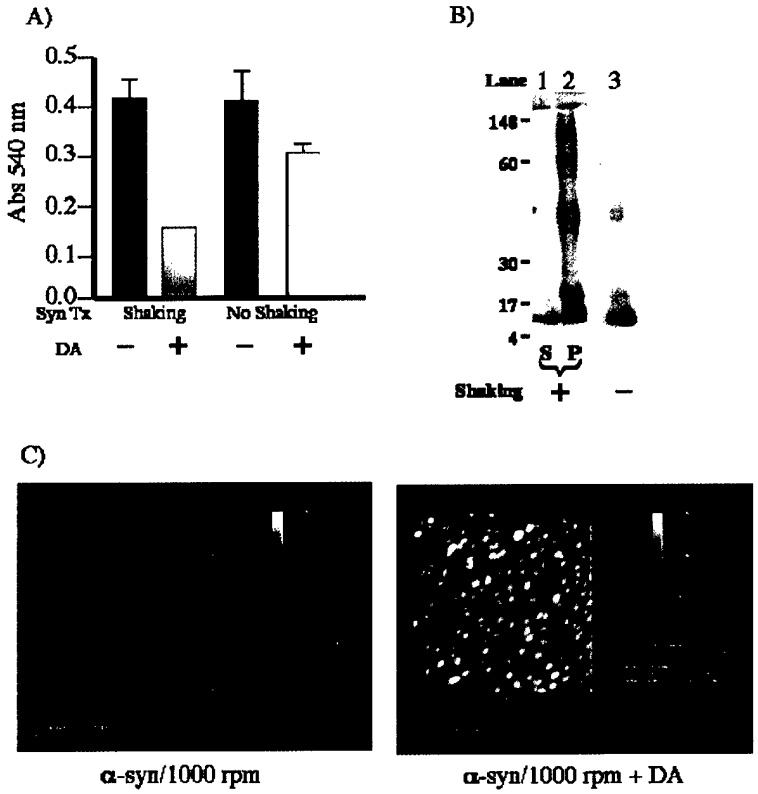

Figure 3. Altered α-syn conformers. Panel A. Congo Red assay. Five μg of protein was incubated with 250 μl of 20 μM Congo Red for 30 min. with gentle shaking. Samples were centrifuged at 13, 000 rpm for 2 minutes and the 540 nm absorbance of the cleared supernatant was determined. Note a decrease in absorbance with the dopamine and shaking paradigm indicates an increase in aggregate formation. **Panel B. α-Syn western blot analysis.** Pellets and supernatants from a Congo Red assay were subjected to PAG-SDS, transferred to PVDF membrane, incubated with anti-α-synuclein antibodies and developed with ECL following incubation with the appropriate secondary HRP-conjugated 2° antibodies. **Panel C. Atomic force microscopy of α-synuclein.** α-Syn subjected to shaking (1000 rpm) in the absence or presence of dopamine followed by AFM analysis. Note the dramatic increase in protein height in the presence of dopamine (white = 20 nm and black = 0 nm).

In this assay, untreated or dopamine treated α-syn was incubated with Congo Red dye followed by centrifugation. Aggregated α-syn binds to the dye with an attendant

decrease in absorbance of the supernatant at 540 nm. Furthermore these Congo Red supernatants (monomeric α-syn) and precipitates (aggregated α-syn) were subjected to polyacrylamide gel electrophoresis under denaturing conditions followed by western blot analysis. As shown in **Figure 3B**, the monomer α-syn resides predominately in the supernatant and can be converted to larger aggregates by shaking. Aggregate formation is greatly favored by incubation with DA (data not shown). Finally atomic force microscopy (AFM) further confirms the formation of large α-syn aggregates following dopamine treatment (**Figure 3C**). These altered forms of α-syn are currently being utilized to screen for conformation specific single-chain antibodies.

I.C. Screening of scFvs. *Rationale: Secreted single chain antibodies directed against definable regions of specific proteins can be produced in large quantities in bacteria.* Using a combinatorial phage display library expressing human immunoglobulin heavy and light chain variable regions, we have screened for phage clones capable of binding to the native α-syn. Panning for antibodies resulted in the identification of two specific scFvs (**Figure 4**). The specific scFvs have been subcloned into bacterial expression vectors and purified by virtue of an incorporated 6-His Tag. Furthermore, purified scFvs have been tested for specificity by enzyme-linked immunoabsorbant assay and purity by western blot analysis.

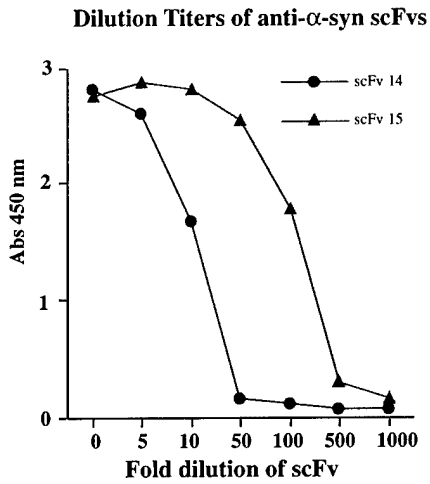

Figure 4. Dilution titers of anti-α-syn scFvs. ScFv 14 and 15 were tested in an ELISA against bacterially expressed and purified α-syn. Note that selected scFvs bind to α-syn in a dose-dependent fashion.

I.D. Characterization of anti-syn single chain antibodies. *Rationale: Single chain antibodies which exhibit high affinity for α-syn conformers, a specific pattern of cellular distribution and varying half-life's can be produced.*

General characterization of scFvs : Quantitative measures of scFvs binding affinity and dissociation kinetics will be determined from plasmon resonance studies with a BIACORE2000. These studies are pending. We are currently panning for scFvs which will specifically recognize dopamine-adducted α-syn.

Conformer specificity: Currently we have identified two scFVs that demonstrate specificity for native α-syn and *do not* interact with α-syn aggregated in the presence of DA (**Figure 5**). We have generated a permuted panel of α-syn peptides spanning the entire polypeptide and have begun to test these scFvs against this panel to identify which linear epitopes are recognized. In addition, we are currently screening against the altered forms of α-syn to identify conformation specific single-chain antibodies.

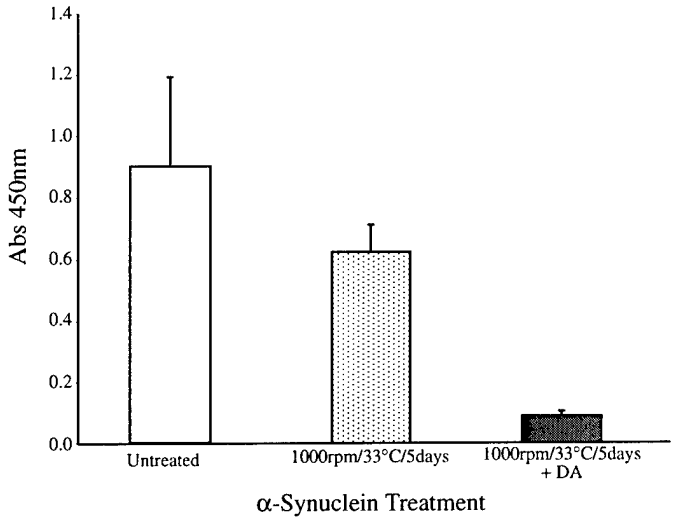

Figure 5. Effect of aggregation on Anti-α-syn scFv activity. Ten μg/ml wildtype α-syn untreated (monomeric α-syn), incubated for 5 days at 1000 rpm/33°C (shaking) or incubated for 5 days at 1000 rpm/33°C in the presence of DA (shaking +) were plated in microtiter wells followed by incubation with bacterially expressed and purified scFv14. Bound scFv was detected by incubation with an anti-HA-HRP antibody. Absorbance measured at 450 nm was recorded.

scFv and antigen protein turnover: The identified scFvs have been subcloned into an HSV amplicon plasmid backbone vector. This vector contains the necessary DNA sequences for efficient viral packaging (ori"S"; packaging "a" site); the HSV IE4/5 promoter followed by a multicloning region for insertion of the structure specific scFv (HSVscFv). The hemagglutinin epitope sequence (YPYDVPDYA; HA tag) has been fused to the C termini of all scFv constructs to facilitate immunolocalization and immunoprecipitation (HSVscFvHA). In the first iteration of this shuttle vector the scFv were subcloned with an upstream secretion signal. These constructs have been expressed in mammalian cells (BHKs) and scFv secretion demonstrated **(Figure 6).**

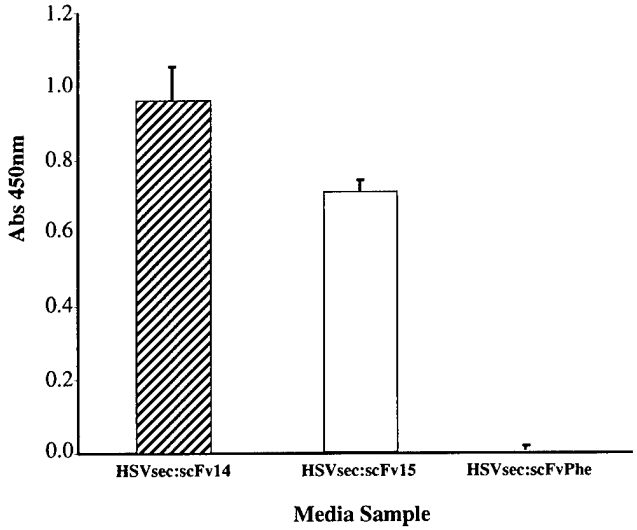

Figure 6. Anti-α-syn scFv ELISA. Ten μg/ml wildtype α-syn was plated in microtiter wells followed by incubation with media from BHK's transiently transfected with HSVsec:scFv14, HSVsec:scFv15 or HSVsec:scFvPhe. Bound scFv was detected by incubation with anti-Myc-HRP antibody and measuring absorbance at 450 nm. HSVsec:scFvPhe is directed against phenobarbital serving as a negative control.

A shuttle vector has been prepared without the secretion signal which will enable efficient transduction of α-synMN9D's or α-synHN33.11's and assessment of intrabody effects on α-syn. These experiments are just beginning and will include studies of scFv and antigen protein turnover and cellular localization by dual-label immunofluorescence.

KEY RESEARCH ACCOMPLISHMENTS

- Wildtype, A53T and A30P α-synuclein have been expressed and purified.
- Doxycycline regulatable cell lines expressing all three types of α-synuclein have been produced.
- Altered conformers of wildtype, A53T and A30P α-synuclein have been produced.
- Single-chain antibodies with specificity for monomeric α-synuclein have been identified, expressed and purified.
- The identified single-chain antibodies have been subcloned into a HSV amplicon shuttle vector and expressed in mammalian cells.

REPORTABLE OUTCOMES

Manuscripts

Maguire-Zeiss KA and Federoff HJ. Convergent Pathobiologic Model of Parkinson's Disease. In: Parkinson's Disease; The Life Cycle of the Dopamine Neuron, 2003; Vol. 991: 152-166; Ann. N.Y. Acad. Sci., Eds. H.J. Federoff, R. E. Burke, S. Fahn and G. Fiskum.

Luo, Y, Henricksen, LA, **Maguire-Zeiss KA**, and Federoff HJ. Development of Nurr1 Stable Cell Lines for the Identification of Downstream Targets. In: Parkinson's Disease; The Life Cycle of the Dopamine Neuron, 2003; Vol. 991: 354-358; Ann. N.Y. Acad. Sci., Eds. H.J. Federoff, R. E. Burke, S. Fahn and G. Fiskum.

Abstracts

Maguire-Zeiss KA, Wuertzer C, Henricksen LA, Venkatesh K, Kang S, Short M, Sullivan M, and Federoff HJ. New York Academy of Sciences Conference, September 18-20, 2002. Parkinson's Disease: The Life Cycle of the Dopamine Neuron. Single Chain Antibodies for PD: Implications for Pathogenesis.

CONCLUSIONS

We hypothesize that in dopamine neurons a steady state exists between the native α-syn, the β-sheet conformer and the aggregated form. Pushing the balance towards pathophysiological protein aggregation will result in cell death. We propose that scFvs which specifically interact with monomer α-syn will reduce the steady state concentrations of oligomers and mitigate toxicity. Thus targeting α-synuclein protein misfolding will enable the development of effective therapy. Toward this goal we have expressed and purified α-synuclein for screening against a combinatorial phage display library expressing human immunoglobulin heavy and light chain variable regions. We have identified two scFvs which preferentially recognize monomeric α-synuclein. We are now testing these scFvs in conditionally overexpressing α-synuclein cell lines to determine where they interfere with α-syn aggregate formation and aid in the dissolution of existing aggregates. Subsequent years will extend these findings *in vivo* to animal models of PD.

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