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TITLE: Interaction of Synuclein and Inflammation in Dopaminergic Neurodegeneration

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Parkinson Disease (PD) is the second most common neurodegenerative disease of our aged population behind Alzheimer’s Disease. Epidemiological, animal and cell culture studies have shown that inflammation is a part of the PD morphological picture. It has been suggested that α-synuclein (a major component of the Lewy bodies present within dopamine neurons of the PD substantia nigra (SN)) is responsible for the observed inflammatory response in the PD brain. We injected α-synuclein and mutated synuclein into the substantia nigra (SN) of rats (8 μg/4μl). We also performed cell culture studies on the activation of microglia by alpha-synuclein. In our in vivo studies, we found that both alpha-synuclein and A53T mutated synuclein both caused apomorphine-induced rotations. The number of rotations were small but significant and indicates that the synucleins caused an increase in sensitivity of the dopaminergic (DA) receptors in the SN. We injected α-synuclein and mutated synuclein into the substantia nigra (SN) of rats (8 μg/4μl). We also performed cell culture studies on the activation of microglia by alpha-synuclein. In our in vivo studies, we found that both alpha-synuclein and A53T mutated synuclein both caused apomorphine-induced rotations. The number of rotations were small but significant and indicates that the synucleins caused an increase in sensitivity of the dopaminergic (DA) receptors in the SN. These studies indicate that there is some interaction between DA and the synucleins. In our in vitro studies, we noted that the synucleins caused microglia to adhere to plastic surfaces. This adhesion was blocked by EDTA which indicated that the process of adhesion in this case was calcium-dependent. In our examination of cell migration using various synucleins, we found that only LTB4, a leukotriene, and CX3CL1, a chemokine, enticed microglia to migrate to naked or laminin-coated filters. While we cannot conclude from our cell culture studies that synucleins can trigger neuroinflammation, we can state that the synucleins can activate microglia and cause them to migrate.

15. SUBJECT TERMS
Synuclein, Parkinson’s disease, neuroinflammation, microglia
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Interaction of Synuclein and Inflammation in Dopaminergic Neurodegeneration, in vivo studies

Introduction
Parkinson’s Disease (PD) is the second most common neurodegenerative disorder after Alzheimer’s Disease (AD) (Fahn and Przedborski, 2009). It is characterized clinically by resting tremor, rigidity, slowness of movement, and postural instability (Fahn and Przedborski, 2009), all attributed to mainly, though not exclusively, the loss of the dopaminergic neurons in the substantia nigra pars compacta (SNpc) and their dopaminergic terminals in the corpus striatum of the nigrostriatal pathway in the brain. Biochemically, PD presents with a profound loss of DA levels in the striatum, which, in part, accounts for the noted motor deficits (Hornykiewicz and Kisk, 1987). Although current therapy includes dopaminergic agonists and cholinergic antagonists, the most reliable and most common therapy remains Levodopa (L-DOPA), a precursor for dopamine (DA) (Fahn and Przedborski, 2009). Neuroinflammation is a newly recognized hallmark of the PD morphological picture (McGeer and McGeer, 2008) and is thought to contribute to DA neuron death in the SNpc and to the progressive nature of PD through glial activation (Fahn and Przedborski, 2009). However, how glial cells and neuroinflammation are involved in PD is not known. It is theorized that the observed activation of glial cells in neuroinflammation may be the cause of the progressive nature of the disease, but this remains to be clarified. We do know that, on autopsy, activated glial cells are found in abundance in the SNpc of PD brains and that there seems to be evidence of an on-going active degenerative process here (Langston et al, 1999). We also know that pro-inflammatory components, such as interleukin 1β (IL-1β) and prostaglandin PGE2, are present in increased levels in the SNpc and in the CSF from PD patients (Mogi et al, 1996). In PD models, not only are activated glia present in the SNpc, also present are markers of inflammation, such as the elevation of NADPH oxidase (Wu et al, 2002), inducible nitric oxide synthase (iNOS) (Liberatore et al, 1999) and PGE2 (Teismann et al, 2003). One interesting finding is the presence of post-translational modified α-synuclein (Przedborski et al, 2001) within the DA neurons of the SNpc. Thus, it is possible that when a DA neuron dies in PD, the extrusion of its contents, i.e. synuclein, into the extracellular space, may trigger not only oxidative stress but also an inflammatory event.

Body of Work

Specific aim-I. Assess the effects of extracellular α-synuclein on glial mobility and proliferation.
Plan: 1) Rats will receive into the substantia nigra a single injection of modified (fibrillar, nitrated, or oxidized) or undified wild-type or PD-linked mutant α-synucleins. Brains will then be harvested and glial cell morphology, topography and density will be compared among each different group. 2) Glial cultures will be exposed to the same set of α-synuclein species and their effects on glia chemotaxis and proliferation will be compared.

Specific aim-II. Examine the stability of extracellular α-synuclein and its effect on glial cell activation.
Plan: 1) The stability and phagocytosis of extracellular α-synuclein species will be compared in vivo (using the SA-IA model) and in vitro (using primary glial cultures) by immunostaining for α-synuclein and glial markers and confocal microscopy, and by an in vitro protein fragmentation assay. 2) The effect of the α-synuclein species on the in vivo and in vitro production of chemokines, cytokines, ROS and NO by glia will also be compared.

Specific aim-III. Define the mechanism by which glial cells recognize α-synuclein.
Plan: 1) Identify the glial receptors involved in the recognition of the α-synuclein species. 2) Characterize the effect of abrogating (knockout mice) and inhibiting (blocking antibodies) glial receptors on endocytosis of and activation by α-synuclein species.
Key Research Accomplishments

During this quarter, we have made advances in both our in vivo and in vitro studies. In our in vivo studies, we noted that both 5.0 micrograms of alpha-synuclein per microliter (total 20 micrograms in 4 microliters) and 2.0 micrograms per microliter (total 8 micrograms in 4 microliters) were highly effective in eliciting an inflammatory response in the SNpc as evidenced by the robust response noted on Iba-1, Cox-2, NFkB, iNOS and GFAP immunostaining. However, both of these concentrations severely damaged the tissues in the SNpc causing them to tear which may be indicative of too strong a concentration of alpha-synuclein. So, in a pilot study to reduce the concentration of the various synucleins enough to elicit a significant inflammatory response and, at the same time not damage the tissues, we decreased the injection concentration of wild-type synuclein, A53T, A30P, and nitrated synuclein to 1 microgram per microliter. Rats (2 per group) received either 4 micrograms (total volume 4 microliters) or 2 micrograms (total volume 2 microliters) of one of the synucleins and were sacrificed at 4 days after injections, the time we demonstrated early on as the peak of the response to the alpha-synuclein injection. Brains were processed for iba-1 immunostaining first and it was noted that only the 4 microliter A30P delivery caused some damage in the tissues including several holes; all 2 microliter deliveries elicited a significant robust microglial response evidenced on iba-1 immunostaining and without damage to the tissues. We believe that we are now at or near the in vivo concentration range of extracellular synuclein, which is somewhere around 250 nm.

Our next step was to dissect the observed events in the inflammation initiation process in relation to PD. In this respect, we found that CD 36, a class B scavenger receptor, is strongly up-regulated at 2-4 days after the injection of wild-type (wt) alpha-α-synuclein into the SNpc of rats. Even though we decreased the concentrations of the synuclein by 80-90%, a strong response in CD36 immunostaining was still elicited. We are now working on the immunostaining method for CD36 in human PD and control tissues using both fresh frozen and paraffin-embedded tissues to ascertain whether or not CD 36 is also up-regulated in the human condition. We are also trying to ascertain in which cell type does this response occur.

Effects of α-synuclein on microglial activation, in vitro studies

The immune response in the brain involves both astrocytes and microglia as these are the sentinel cells that react to the presence of foreign proteins. Along with the change in the physical appearance of these cells, there is also a change in the cell’s surface and surface antigens that probably govern some of those responses to foreign body intrusions. Many surface receptors have been implicated in the inflammatory response. But, we noted that since the class B scavenger receptor CD36 has already been connected to the microglial response in other neurodegenerative diseases (Coraci et al, 2002, El Khoury et al 2003; Medeiros et al, 2004), why should PD, a neurodegenerative disease, be excluded. Accordingly, we embarked on a series of in vitro studies.

Adhesion studies:
Role of CD36 and CD11b: These studies have identified two specific microglial cell surface receptors that mediate murine BV2 microglial cell interactions with extracellular alpha-synuclein. Antibodies that block the CD18 family of integrins and the pattern recognition receptor CD36 reduce the capacity of: 1) BV2 murine microglial cells, 2) N9 murine microglial cells and 3) primary neonatal murine microglial cells to adhere to surfaces coated with laminin and alpha-synuclein, but have no effect on the interaction of these cells to surfaces coated with laminin alone. Moreover, N9 and BV2 cells exhibit a 25-50% increase in adhesion capacity on alpha-synuclein coated surfaces as compared to laminin coated surfaces. Several other results have been generated to confirm the role of CD36 in microglial interactions with alpha-synuclein. We show, for example, that genetically-modified CHO cells that express CD36 surface receptors increase their capacity, several folds, to bind to alpha-synuclein-coated surfaces. The increase in the adhesion of transgenic CHO CD36+/+ to alpha-synuclein is blocked by anti-CD36 antibodies and not by CD11b antibodies (note that CHO cells do not express CD11b either). Other
control experiments show that blocking antibodies directed against CD36 and CD11b have no effect on the adhesion of murine primary astrocytes to α-synuclein-coated surfaces. We have also obtained, in collaboration with Dr. Joseph El Khoury from Harvard Medical School, primary adult murine microglial cells from mice that lack CD36. These cells have been immortalized with an adenovirus. Using these CD36−/− microglial cells we observed no increase in cell adherence to alpha-synuclein-coated matrices as compared to laminin coated surfaces. As expected, anti-CD36 antibodies also had no effect on the adherence of CD36−/− microglial cells to either laminin or alpha-synuclein whereas preliminary experiments show that anti-CD11b antibodies block their adherence. In summary, these data support our hypothesis that CD36 and CD11b receptors mediate interactions of microglial cells with alpha-synuclein matrices.

Effects of other pattern recognition receptors:
The fact that neither antibody alone or in combination inhibits more than 50% of cell adhesion prompted us to examine other pattern recognition receptors. We now see that both anti-RAGE antibodies and anti SR A1 antibodies block N9 microglia cell adhesion to alpha-synuclein by 30% and 40%, respectively. Thus, there are a variety of pattern recognition receptors (PRR) that are involved. We are currently testing the effects of multiple anti-PRR antibodies to better understand whether there is an additive or synergistic effect. We will also examine the effects of treating N9 cells with anti-CD11b and anti-RAGE or anti-CD11b and anti-SRA-1 antibodies to assess possible synergistic effects on cell adhesion. Future experiments are underway to examine the role of both integrins and PRR receptors in the adhesion of microglial cells with mutated and modified forms (nitrat ed, oxidized or aggregated alpha-synuclein). Preliminary results already have shown some differences in the role of these receptors with the mutated or modified forms of alpha-synuclein.

Chemotaxis
The recruitment of microglial cells to DA SN neurons in PD is a hallmark of the disease process. We therefore developed a chemotaxis assay to explore the role of these receptors in microglial migration to sites of laminin and alpha-synuclein deposition. We hypothesize that the receptors we have identified above are critical in microgliosis recruitment to extracellular deposits of alpha-synuclein in the CNS. In our assay, we coat BD cell culture trans-wells with the respective matrix proteins and then add the microglial cells to the upper chamber. We then add a chemoattractant in the lower compartment and follow cell chemotaxis into the lower compartment. We already know that BV2 cells do not migrate well using this assay, so we switched to utilizing N9 murine microglia cells. While N9 cell migrate better than BV2 cells, only a very small percent <1% of these cells will migrate across the porous membrane and remain attached to the underside of the membrane. To detach those adherent cells, we simply add trypsin/EDTA for 5 min, and allow the cells to drop into compartment of the trans-wells without promoting any of the non-migratory cells in the upper compartment from migrating across the membranes. This assay is working quite well.

To increase the number of migratory cells, we have isolated a subpopulation of N9 cells that are 5-fold more migratory in response to specific chemoattractants. This was done by growing those N9 cells that had migrated across laminin in response to a chemoattractant. Thus far, we have subpopulations of N9 cells that specifically chemotax in response to G M C S F, MCP-1, TNF and IL-2. We have now begun to assess the migratory properties each of these subpopulations of N9 cells. Using GMCSF-migratory responsive N9 cells, for example, we show that they migrate (>5% migrate/24 h) much better than normal N9 cells (1% migrating per 24h) across laminin coated trans-wells in response to GMCSF. However, the presence of alpha-synuclein reduces migration across these trans-wells to less than 0.5%. Equally important, we show that only anti-CD11b and not anti-CD36 reverses this inhibition of migration. In the presence of anti-CD11b almost 4% of the cells migrate across trans-wells coated with both alpha-synuclein and laminin.

Future experiments are planned to expand these findings to examine the effects of adding both anti-CD11b and anti-CD36 antibodies on chemotaxis using the various subpopulations we have obtained. In addition we will examine chemotaxis of immortalized primary microglial cells lacking CD36, CD11b
or double knockouts. Finally, we have recently isolated a subpopulation of GMCSF-migratory responsive N9 cells that have migrated across trans-wells coated with both alpha-synuclein and laminin. We use FASC analysis to examine their cell surface receptors to assess whether these cells are deficient in CD36, CD11b or both. These experiments highlight the usefulness of utilizing N9 cells in identifying the various cell surface receptors mediating chemotaxis.

**Production of inflammatory cytokines**

As an indicator of the inflammatory processes involved in PD, there is an increase in a number of inflammatory cytokines produced in PD patients and found in CSF. We have therefore assessed the capacity of N9 cells to produce TNF after adhering to various matrices. For these experiments we have used commercial ELISAs which are highly sensitive assays for TNF production and N9 cells. We show that culturing N9 cells for up to 24 hours on laminin coated surfaces produces 48 pg/100,000 cells within 24 hr. However, maintaining N9 cells for 24 on surfaces coated with both alpha-synuclein and laminin increase TNF production to 210 pg/100,000 cells.

We plan to examine the effects of anti-integrin (CD11b) antibodies or anti-pattern recognition receptor antibodies on TNF production as well as examining TNF production by N9 cells maintained on surfaces coated with mutated or modified synuclein. Preliminary data show that N9 cells cultured on plates coated with laminin and A-30P mutated alpha-synuclein increases TNF production another two-fold increase (406 pg/100,000 cells) over cells maintained on alpha-synuclein matrices and almost a 10 fold increase in TNF production over cells cultured on laminin surfaces.

**ROS Production**

Increased ROS production by microglial cells is a common feature in a variety of neurodegenerative diseases. We therefore are developing assays to assess ROS production by N9 cells interacting with alpha-synuclein coated matrices. Initially we have been using an NBT-based assay to assess ROS production. This is still work in progress but preliminary results indicate that ROS production is undetectable when N9 cells adhere to laminin matrices but appears robust when N9 cells adhere to alpha-synuclein matrices. As a control we show that N9 cells produce robust amounts of ROS when they adhere to oxidized LDL coated matrices. We still have to work out a better way to quantitate the ROS produced.

**Reportable Outcomes**

In summary, we have designed a systematic approach to identifying candidate receptors that mediate the interaction and functions of microglial cells with native, mutated, and modified forms of alpha-synuclein. In addition, we have begun to generate various transgenic microglial cells (such as CD36 and CD11b knockouts) to examine their properties in vitro as well as using these mice to examine the microglial response to the varied synucleins in an in vivo setting. Our unpublished results showing that the absence of CD36 or the use of agents that block CD36 impacts the development of Alzheimer’s disease in mouse models suggest that these agents may play a therapeutic role in PD as well.

**Conclusions**

We conclude that the synucleins can initiate a neuroinflammatory response in the extracellular space and microglia are important players here. Further, from our in vivo studies, we think that CD36 and CD11b may be early responders to the presence of extracellular synuclein, thus early initiators of this response. In breaking down the inflammatory response to synucleins into single events, from our in vitro studies, we now know that PRR are necessary for this inflammatory response as is cytokine and chemokine up-regulation and chemotaxis between the initiator (synuclein) and the initiatees (cytokines, chemokines, PRR).
References