Research article

Re-circulating Phagocytes Loaded with CNS Debris: A Potential Marker of Neurodegeneration in Parkinson's Disease?

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Abstract: Diagnosis and monitoring of diseases by measurement of biochemical markers has most commonly been performed on samples of peripheral blood. However, no such markers are available for clinical use in the major diseases of the central nervous system (CNS). In Parkinson's disease circulating biomarkers would find clinical utility in early diagnosis and also monitoring of disease progression. Of particular interest is early diagnosis as this would create .a window of opportunity for treatment with neuroprotective drugs. We have developed a novel strategy for monitoring disease activity in the CNS based on the recognition that tissue injuries incite inflammation and recruitment of phagocytes that engulf debris. We postulated that some of these debris laden phagocytes may return to the peripheral blood and their cargo of CNS proteins could be measured. If CNS antigens can be measured in PBMCs it may be an indicator of active neurodegeneration as the debris engulfed by phagocytes is completely degraded within days. To make this approach more specific to Parkinson's disease we probed PBMC lysates for neuromelanin as a marker of degeneration within the substancia nigra. We performed a proof of principle study in ten subjects with early PD and ten age and sex matched controls. The biomarkers neuromelanin, Tau protein, UCH-L1 and HPCAL-1 were measured in PBMC lysates from these two groups. Neuromelanin and Tau protein mean levels were elevated in PD compared with controls and was extremely statistically significant in both cases. UCH-L1 and HPCAL-1 mean levels were elevated in PD over controls and were not quite significant in both cases. These results suggest that this is a promising new approach for diagnosis and monitoring of PD and potentially other CNS diseases.

Abbreviations: PD – Parkinson's disease; PBMC – peripheral blood mononuclear cells; UCHL1 – ubiquitin carboxyl-terminal esterase L1; HPCAL-1 – hippocalcin like protein 1; HRP – horse radish peroxidase; CNS – central nervous system; SN – substantia nigra; REM – rapid eye movement; PBS

– phosphate buffered saline; BSA – bovine serum albumin

1. Introduction

PD is associated with a loss of neurons, particularly in the Substantia Nigra (SN), whose neurons produce dopamine. Loss of dopamine results in inappropriate neuronal signaling, causing many of the clinical characteristics of PD, particularly those related to motor function. Neuronal degeneration is not limited to the SN, though it is grossly the most evident site. PD is a chronic neurodegenerative disorder involving loss of neurons in various regions of the brain. It is clinically characterized by resting tremor, bradykinesia (a slowness in the execution of movement), and rigidity, often accompanied by postural instability. Motor symptoms represent the most widely recognized clinical diagnostic of PD. Since the appearance of these symptoms correlates with a high degree of neural degeneration [1,2], it is too late to expect any potential neuroprotective therapies to be effective. To facilitate neuroprotective therapies it will be necessary to identify individuals that are developing PD in a prodromal (pre-motor) phase of the disease. This would identify a window of opportunity for prevention of overt PD development.

Non-motor clinical symptoms, such as hyposmia, REM behavior disorder and constipation precede motor symptoms. While these factors could be used in combination to identify a high risk prodromal stage, they are not diagnostic in themselves and could represent a variety of other pathologies. Of these symptoms, olfactory assessment does seem to offer a relatively high degree of both sensitivity and specificity and as such could represent a low-cost, easy identification of at-risk individuals appropriate for further assessment [1,2]. There are currently no fluid based biochemical markers for Parkinson's disease in clinical use.

Tissue injuries usually result in some degree of inflammation involving infiltration with phagocytic cells that engulf and remove debris from the site of injury [3]. We have postulated that some of these debris laden cells may return to the peripheral blood circulation and by probing for components of the debris we may have a biomarker strategy to monitor neurodegeneration. Furthermore, as the function of these phagocytes is to breakdown the debris, detection of debris components in re-circulating phagocytes indicates a process that is actively happening as the debris is undetectable after a few days [4]!

Here we report the results of a pilot study using this approach to detect CNS components in PBMC lysates from recent onset Parkinson's disease and age and gender matched apparently healthy controls.

2. Materials and Method

2.1. Human Samples

2.1.1. Blood

Blood samples from ten apparently healthy controls and ten Parkinson's disease patients were obtained from Sanguine biosciences (Sherman Oaks, CA) for this pilot study. Blood samples were drawn with informed consent under an IRB approved protocol. Early stage Parkinson's disease

patients were selected by duration since diagnosis (less than 3 years) or early stage of disease based on the Hoehn and Yahr Scale [5]. Apparently healthy controls were age and gender-matched to the Parkinson's patients selected (Table 1).

Table 1. Patient and Control Subject Characteristics.

	Control	**PD**	**P value**
Age (s.d.)	67.1 (7.9)	61.7 (13.4)	0.2868
Age of Diagnosis (s.d.)	N/A	58.6 (12.8)	–
Years Since Diagnosis (s.d.)	N/A	3.1 (2.3)	–
% Male	60.0	60.0	1.0000

2.1.2.   Human Substantia Nigra Frozen Sections

5um acetone fixed frozen sections of normal substantia nigra from a 72 year old female at autopsy were obtained from Biochain Institute Inc. (Newark, CA). Tissues were collected under an IRB approved protocol.

2.2. Antibodies and Peptides

Biotinylated 4B4 peptide was synthesized by the Tufts University Core Facility peptide synthesis service (Tufts University College of Medicine; Boston, MA). The 4B4 sequence was previously reported [6,7]. Rabbit anti human Tau protein was purchased from Dako Corp.(Carpenteria, CA). Mouse monoclonal anti UCH-L1 was purchased from (Santa Cruz Biotechnology, Inc.; Dallas, Texas) and Rabbit anti Hippocalcin like 1 was purchased from (Life Span Bioscience Inc.; Seattle, WA). HRP conjugated Streptavadin was purchased from Thermo Scientific (Rockford, IL). HRP conjugated anti mouse and anti rabbit IgG were purchased from Santa Cruz Biotechnology Inc. (Dallas, Texas).

2.3. Melanin Staining Kit

A Fontana-Masson stain kit was obtained from American Master Tech Scientific Inc. (Lodi, CA).

2.4. Staining Frozen Sections

Fontana-Masson staining was performed according to manufacturer's instructions.

4B4 peptide was diluted in PBS + 1% BSA and incubated on frozen sections for 60 minutes. The sections were then washed 3 times with PBS before incubation with a 1:500 dilution of HRP-streptavadin (KPL; Gaithersburg, MD) in PBS + 1% BSA for 60 minutes. The sections were then washed 3 times with PBS before incubation with TMB insoluble substrate (EMD: Millipore; Billerica, MA). Sections were counterstained with Nuclear Fast Red Results and coverslips mounted prior to microscopy. The stained sections were visually scored and photomicrographs taken.

2.5. Human Peripheral Blood Mononuclear Cells

PBMCs were prepared from whole blood samples collected in BD VacutainerR Cell Preparation Tubes (CPTTM) with Sodium Citrate as anticoagulant (Beckton, Dickinson and Co; Franklin Lakes, NJ), collecting two tubes per patient. The tubes were centrifuged at 3000 rpm for 30 minutes. The PBMCs from each tube were collected and washed two times in 45mL Phosphate Buffered Saline (PBS), by centrifugation at 1500 rpm for 15 minutes for each wash. The supernatant was aspirated and discarded. The remaining pellets were recombined and hypotonically lysed with 500 μL deionized water. The suspension was brought to isotonic with 10x PBS, and 10 μL protease arrestTM (G-Biosciences; St. Louis, MO) was added. Aliquots were stored at −80 °C. All samples were frozen for less than one month prior to assay.

2.6. Protein Quantification

Protein concentrations in PBMC lysates were measured using the Bradford assay (BioRad Laboratories; Hercules, CA) with Bovine Serum Albumin (BSA) as the standard. Standards of 5 to 25 micrograms per mL of protein were prepared in 0.8 mL of PBS. Unknowns were diluted as necessary in PBS. 0.2 mL Coomassie Brilliant Blue dye reagent (Bio-Rad) was added to each tube, vortexed and incubated 5 minutes. Absorbance was measured at 595nm. Unknowns were quantified by interpolating absorbance against the standard curve. HRP conjugated anti mouse IgG and HRP conjugated anti rabbit IgG were purchased from Santa Cruz Bioscience (Dallas, Texas).

2.7. 4B4 Peptide Binding Assay

Human PBMC lysate was normalized to 5 μg/mL protein concentration in 0.5 M NaOH and adsorbed directly onto a polystyrene plate. Wells were coated with 100uL each of lysate dilution, in duplicate or triplicate, then incubated at 60 °C for two hours, followed by 30 minutes at 80 °C. Wells were washed four times with deionized water and blotted. Wells were blocked with 200 μL per well of 1% BSA/0.1 M Glycine in PBS and incubated for 2 hours at room temperature or 4 °C overnight. Wells were washed four times with PBS and blotted. 100uL per well of 0.1 μg/mL biotinylated 4B4 peptide diluted in 1% BSA-PBS was added and incubated for 2 hours at room temperature. Wells were washed four times with PBS and blotted. 100 μL per well of streptavidin-HRP diluted 1:500 in 1% BSA-PBS was added and incubated 1 hour at room temperature. Wells were washed four times with PBS and blotted. 100 μL per well of TMB substrate solution was added and incubated 30 minutes at room temperature. Added 100 μL per well of TMB stop solution and read using BioTek ELx800 plate reader at 450 nm.

2.8. Direct ELISA

2.8.1. Tau and Hippocalcin-like 1

PBMC lysate was adjusted to 5 μg/mL protein concentration in PBS and adsorbed onto a polystyrene plate. Wells were coated with 100 μL each of lysate dilution, in duplicate or triplicate, then incubated for two hours at room temperature. Wells were washed four times with PBS and

blotted. Wells were blocked with 200 μL per well of 1% BSA/0.1M Glycine in PBS and incubated for 2 hours at room temperature or 4C overnight. Wells were washed four times with PBS and blotted. Added 100uL per well of 1:100 antibody (rabbit anti-human-Tau or anti-human-HPCAL1) diluted in 0.1% BSA/0.05% Tween20 in PBS, and incubated for 2 hours at room temperature. Wells were washed four times with PBS and blotted. Added 100 μL per well of 1:5000 anti-rabbit-IgG-HRP diluted in 1% BSA-PBS and incubated 1 hour at room temperature. Wells were washed four times with PBS and blotted. Added 100 μL per well of TMB substrate solution and incubated 30 minutes at room temperature. Added 100 μL per well of TMB stop solution and read using BioTek ELx800 plate reader at 450 nm.

2.8.2. UCH-L1

PBMC lysate was adsorbed onto a polystyrene plate and blocked as described above. 100 μL per well of 1:100 mouse anti-human-UCH-L1 diluted in 0.1% BSA/0.05% Tween20 in PBS was added and incubated for 2 hours at room temperature. Wells were washed four times with PBS and blotted. 100 μL per well of 1:5000 anti-mouse-IgG-HRP diluted in 1% BSA-PBS was added and incubated 1 hour at room temperature. Wells were washed four times with PBS and blotted. Color development and measurement was as described above.

2.9. Statistical Analyses

Results from the peptide binding assay and ELISA assays were analyzed for statistical significance using the Graphpad QuickCalcs t-test calculator (Graphpad Software Inc.; La Jolla, CA).

3. Results

Acetone fixed frozen sections of human SN were stained for neuromelanin granules with the Fontana-Masson stain (Figure 1, left) and revealed black staining of neuromelanin containing cells in the SN. Staining of these sections with biotinylated 4B4 peptide revealed a similar distribution of blue stained granules (Fig1-right).

PBMC lysates of PD subjects and controls were probed for the presence of CNS antigens by peptide binding assay (for neuromelanin) and ELISA (Figure 2). Eight of 10 PD subjects had levels of 4B4 binding above the control subjects (Figure 2, top left); $p = 0.0082$ (Table 2). Seven of 10 PD PBMC lysates had levels of Tau protein above the highest control (Figure 2, top right); $p = 0.0047$ (Table 2). Hippocalcin like1 was elevated above the highest control value in 6 of 10 PD subjects (Figure 2, bottom left); $p = 0.0521$ (Table 2) and UCH-L1 was elevated above the highest control value in 5 of 10 PD subjects (Figure 2, bottom right); $p = 0.066$ (Table 2).
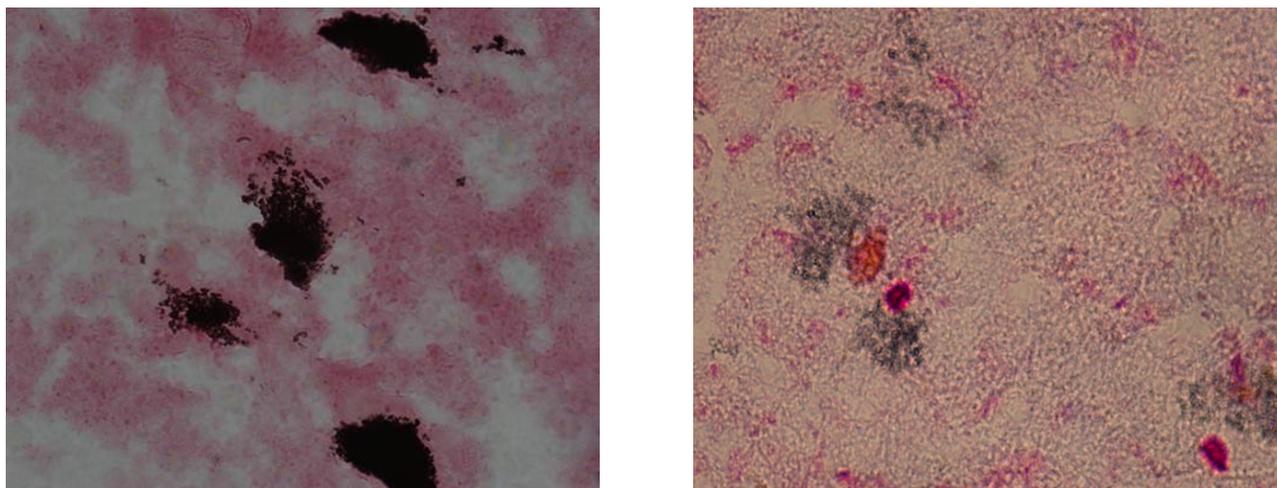
Figure 1. Fontana-Masson Staining (left) and 4B4 Peptide Staining (right) of Human Substantia Nigra.
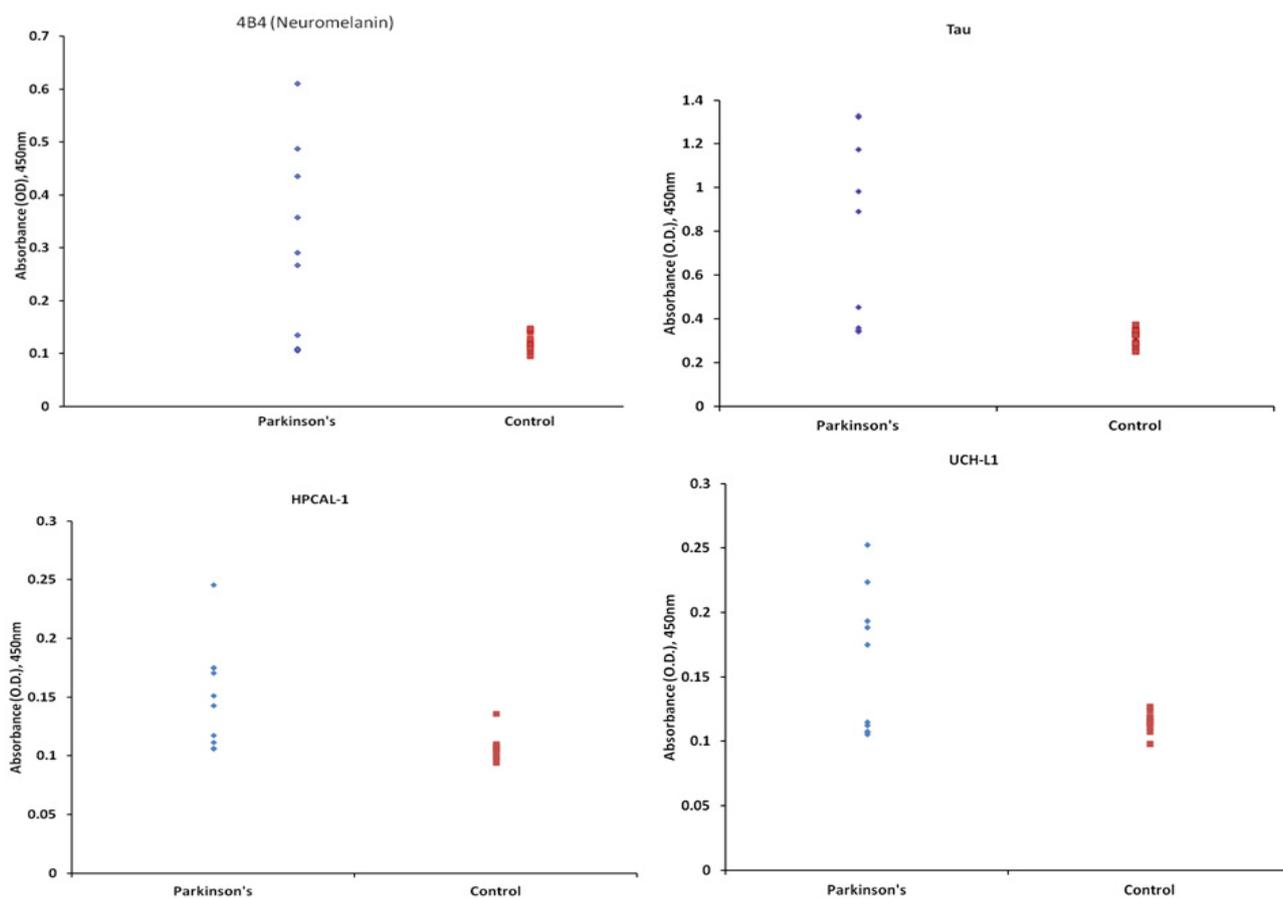

Figure 2. CNS Components in 10 PD subjects and 10 Controls. *Top Left*: **Neuromelanin;** *Top right*: **Tau protein;** *Bottom left*: **Hippocalcin like-1;** *Bottom right*: **UCH-L1.**

Table 1. Summary of results in human control and PD samples.

Component	Control	PD	*p* value
Neuromelanin (4B4), OD450 (s.d.)	0.12 (0.02)	0.29 (0.18)	0.0082
UCH-L1, OD450 (s.d.)	0.12 (0.01)	0.16 (0.06)	0.0521
Tau, OD450 (s.d.)	0.32 (0.04)	0.76 (0.43)	0.0047
HPCAL1, OD450 (s.d.)	0.11 (0.01)	0.15 (0.04)	0.066

4. Discussion

The 4B4 peptide has previously been shown to bind to microbial melanins and eumelanin in human skin [6,7] and we have now demonstrated, by histochemical staining, that it also binds to neuromelanin in the substantia nigra of the human brain. This enabled us to use the 4B4 peptide as a reagent for detection of neuromelanin in phagocytes within PBMCs. We found, in this study, that the mean 4B4 binding levels to PBMC lysates in recently diagnosed PD subjects and age and gender matched controls were statistically significantly different by t-test and that eight of the ten PD subjects had a 4B4 binding level that was above the highest control. This would imply that 4B4 binding may be a useful marker of PD.

What of the two PD subjects that were negative? Is it possible that they were misdiagnosed and have an atypical presentation of essential tremor? A DAT scan could answer this question but that data was not available as these blood samples were obtained from a commercial bio-banking source. This does, however, raise the intriguing possibility that a simple and inexpensive blood test may be able to differentiate PD from other movement disorders and aid in obtaining a correct diagnosis. It is also of interest to note that the two PD subjects that were in the control range for neuromelanin also measured in the control range for Tau protein, HPCAL1 and UCH-L1.

We also analyzed these lysates for the presence of other CNS antigens. The Tau protein has been implicated in the pathogenesis of neurodegenerative diseases [8] and may be present in phagocytosed neuronal debris. We found that the mean levels of Tau protein in PBMC lysates in recently diagnosed PD subjects and age and gender matched controls were statistically significantly different by t-test and that seven of the ten PD subjects had a Tau protein level that was above the highest control. This would also imply that Tau protein in re-circulating phagocytes may be a useful marker of PD.

Hippocalcin like 1 is a calcium sequestering protein and is a member of the neuron specific calcium binding proteins family localized to the brain and retina [9]. Hippocalcin like-1 may contribute to the calcium-dependent regulation of rhodopsin phosphorylation and may be of relevance for neuronal signaling in the CNS. We analyzed these PBMC lysates for the presence of Hippocalcin like-1 protein. We found that the mean levels of Hippocalcin like-1 protein in PBMC lysates in recently diagnosed PD subjects and age and gender matched controls were not quite statistically significantly different by t-test, however is only two significant figures are considered then the p value is 0.05 which is conventionally accepted as statistically significant. Six of the ten PD subjects had a Hippocalcin like-1 protein level that was above the highest control. This would also imply that Hippocalcin like-1 protein in re-circulating phagocytes may be a useful marker of PD.

Ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) is a de-ubiquitinating enzyme. UCH-L1 expression is specific to neurons and to cells of the diffuse neuroendocrine system and their tumors. It is present in all neurons (accounting for 1–2% of total brain protein) [10]. The UCH-L1 gene has been associated with both PD and Alzheimer's disease [11–13].

We found that the mean levels of UCH-L1 protein in PBMC lysates in recently diagnosed PD subjects and age and gender matched controls were not quite statistically significantly different by t-test. Five of the ten PD subjects had a UCH-L1 protein level that was above the highest control. This would similarly imply that UCH-L1 protein in re-circulating phagocytes may be a useful marker of PD.

These results imply that debris loaded macrophages return to the peripheral blood circulation. This may be a direct re-entry via local capillaries. Evidence that this may be so was reported in a rodent model of retinal degeneration [14] in which it was observed by electron microscopy that macrophages loaded with photoreceptor debris were re-entering local capillaries.

Monocytes/macrophages are recruited to sites of injury by chemotaxis along gradients of secreted chemokines and this must be occurring in PD. The recent report that severity of PD is associated with circulating level of the chemokine CCL5 is of extreme interest [15]. In this study CCL5 was measured in sera of PD subjects at different Hoehn-Yahr stages. Regression analysis revealed an association of CCL5 level with PD progression although it was not a strong association. The regression coefficient was 0.362 indicating that the association is responsible for about one third of the variation in the data and was extremely statistically significant ($p = 0.001$). An earlier study did not find any association of PD severity with serum levels of the chemokines CCL3, CCL11, CCL24, CXCL8 and CXCL10 [16]. Consequently, progression of PD is likely to be due to multiple mechanisms but infiltration of immune cells under the influence of CCL5 may play a significant but not exclusive role.

5. Conclusion

The prevalence of elevation of marker levels in PBMC lysates over controls was highest for 4B4 binding at 80%, however the lower prevalence of the other markers does not disqualify them from consideration as they may make useful contributions to a biomarker panel and algorithm that could potentially be used for both early diagnosis of PD and monitoring of disease progression.

Acknowledgments

Conflict of Interest

Vanessa White and Ramesh Nayak are both employees of MSDx, Inc. Ramesh Nayak is a share holder in MSDx Inc.

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