ADNI Alzheimer's Disease Neuroimaging Initiative

Redox-reactive Antiphospholipid Autoantibodies: Early Stage Alzheimer's Disease Blood Biomarkers

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Contents

Summary

Redox reactive autoantibodies (R-RAA) represent a novel family of antibodies detectable only after exposure of serum, plasma or immunoglobulin fractions to oxidizing agents. We have previously reported that certain R-RAA, specifically antiphospholipid antibodies (aPL), are significantly decreased or absent in the cerebrospinal fluids (CSF) of autopsy-confirmed Alzheimer's disease (AD) patients. The documented increased oxidative stress that resides in AD patients results in oxidation of proteins, lipids and DNA. This can be attributed in part to the abnormal enrichment of redox reactive metals in AD patient brains. In the current communication, we report our findings measuring R-RAA aPL in 3 cohorts of individuals: 30 patients with mild cognitive impairment, 30 patients with Alzheimer's dementia and 30 age-matched volunteer controls, all obtained from the ADNI group. The sera from the MCI population contained significantly elevated R-RAA activity in contrast to AD patient and/or control sera. From these data we propose that longitudinal testing of an individual for increases in R-RAA activities over a previously established baseline may serve as a sero-epidemiologic blood biomarker for early onset dementia of the Alzheimer's type.

Methods

Serum samples. Frozen serum samples (0.5 ml) were provided by the Alzheimer's Disease Neuroimaging Initiative (ADNI). The samples originated from 3 cohorts of individuals (30 patients diagnosed with mild cognitive impairment, 30 patients diagnosed with Alzheimer's dementia and 30 age-matched volunteer controls). The samples were randomized and coded by the ADNI such that the investigators performing the analysis were blinded to the sample origin.

Hemin antibody unmasking: >99% pure hemin was purchased from Frontier Scientific Inc. (Logan, UT). Experimental conditions for using the Frontier hemin were established wherein the incubation times, temperatures, pH and dilutions to achieve optimal R-RAA activities**.** Aliquots of the 90 ADNI serum samples stored at -80^{o}C were thawed and treated with Cleanascite^{TM} (Biotech Support Group Inc. North Brunswick, NJ) 1:4 vol/vol in a 2 ml micro tube. The samples, rocked at 37^{o}C for 10 min were then centrifuged for 1 min. at 16,000 × g. A ~130mM hemin solution was made by dissolving hemin powder in 1 M NaOH with warming and vigorous stirring. The solution was cooled to room temperature and filtered through a 0.45 micron filter. The stock hemin concentration was determined by using the extinction coefficient of hemin in NaOH; 58,400 cm^{-1} M^{-1} at 385 nm (*1*). The stock hemin solution remains stable for at least 6 months at 4^{o}C.

An aliquot of the stock hemin solution was added to a buffer containing 20 mM Tris base,151 mM NaCl, with 3mM NaN_3 and the pH adjusted to 7.8 by using 1M HCl (final hemin concentration = 1.28 mM). A control buffer without hemin was prepared using the same volume of 1M NaOH and adjusted to pH 7.8 as above. Serum aliquots from each donor were diluted to a final of 1:15 vol/vol in the hemin and control TBS buffer and placed on a rocking table for 3 hr at 37^{o}C then frozen at -80^{o}C prior to ELISA analysis.

aPL ELISA: The thawed samples were diluted in bovine serum albumin (BSA) and adult bovine plasma (ABP) as previously described (*2*) to a final serum dilution of 1:50 for ELISA analyses. The final concentrations of BSA and ABP were 1% and 10%, respectively. The buffers supplemented with ABP provide plasma proteins that after binding to the phospholipids undergo a conformational change that becomes the target for the autoantibody (*3*). The most common plasma protein to bind cardiolipin is β- 2 glycoprotein and prothrombin whereas phosphatidylethanolamine binds high and low molecular weight kininogens (*4*). In the BSA supplemented buffer, the autoantibodies recognize the phospholipid independent of other plasma proteins (*5*). Three aPL isotype specificities (IgG, IgM and IgA) were assessed for reactivity against phosphatidylserine (PS), cardiolipin (CL), phosphatidylethanolamine (PE) and phosphatidylcholine (PC). Standard curves were generated for each aPL Ig isotype using standardized aPL antiserum, and OD values for the hemin treated and non-treated samples were interpolated from the calibration curves. The R-RAA activity (in OD units) was expressed as the OD difference between hemin treated and non-treated data points for each sample.

Statistical analysis of data: The combined data sets were then grouped into their respective 3 cohorts after receipt of the assignment codes from ADNI. (Normal age-matched controls and patients diagnosed either with mild cognitive impairment or Alzheimer's dementia). First, the

Kruskal-Wallace algorithm was used to identify significant differences between groups across all data sets. The non-parametric Mann-Whitney U Test (SPSS version 16, Chicago Illinois) was used to test the null hypothesis that the distributions of group OD's were equal. The results with their associated errors and p-values are shown in figure 1.

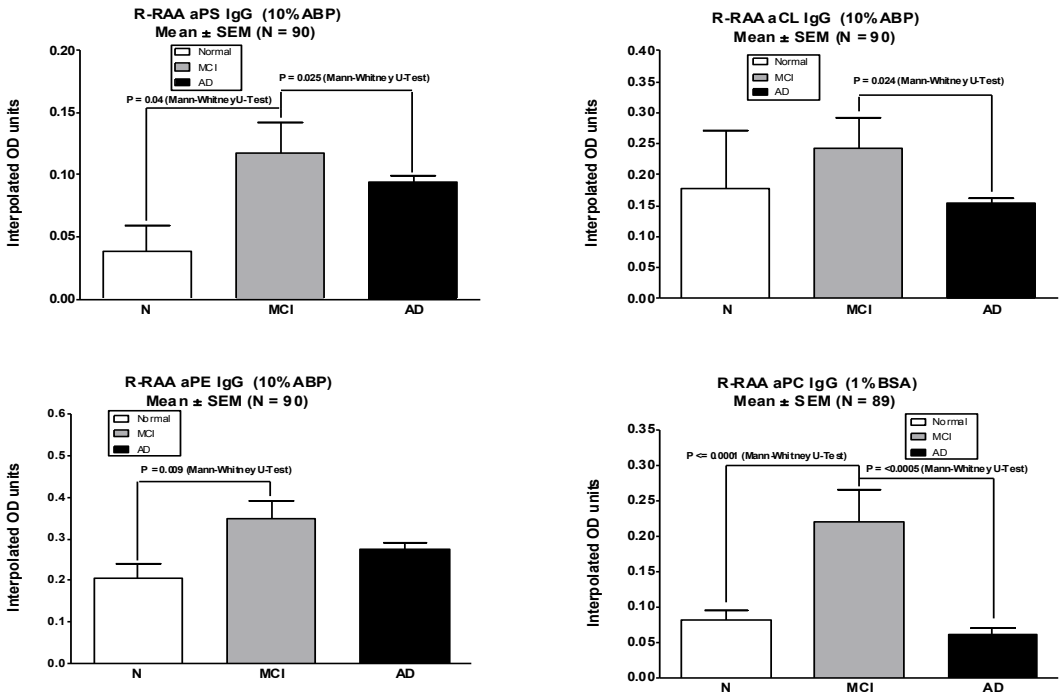

Figure 1: *Hemin unmasked redox reactive antibody activity in sera from normal age-matched controls and patients diagnosed either with mild cognitive impairment or Alzheimer's dementia. ELISA OD values are interpolated from standard curves using qualified lots of anti-phospholipid antisera. Intrinsic antibody activity (OD values of the same serum samples prepared and analyzed identically, but in the absence of hemin) is subtracted from the hemin-treated OD values to give the R-RAA aPL OD units in the serum samples reported in the figure.*

Results and Discussion

It was previously demonstrated in a smaller study (*6*) that R-RAA aPL activity is increased in the serum of patients diagnosed with mild cognitive impairment. The data presented in this study corroborates this finding with a more robust difference due to increased sample size. It was also noted in the earlier small study that R-RAA aPL serum analysis could discriminate sera from patients diagnosed with AD. We could not replicate this observation in the current study. During the course of these investigations, we demonstrated that the source and purity of the hemin influence R-RAA aPL results. We are now using a highly purified and characterized grade of hemin and fully expect inter-assay results to be comparable in future studies.

We are now confident that the levels of R-RAA aPL in serum increase in patients that exhibit signs of mild cognitive impairment and that these levels fall as fulminate disease ensues. There is

precedence of increased biological activities in the MCI population that are markedly different from cognitively normal individuals or late stage AD patients. For example, DeKosky and colleagues (*7*) have documented the up regulation of choline acetyltransferase activity in the hippocampus and frontal cortex of elderly subjects demonstrating MCI. Bruce-Keller and co-workers (*8*) have reported significant elevations in NADPH oxidase (NOX) activity in the temporal gyri of MCI patients. There are also confirmed studies to show that oxidative stress, in both brain and peripheral tissues, is one hallmark of early stage AD in cognitively impaired patients (*9*), (*10*). Of special interest are studies that document increased redox-reactive iron in the brains, CSF and peripheral tissues of MCI patients which correlates with an accumulation of free radical damage and parallels closely to the degree of cognitive impairment in these subjects (*11*). In the laboratory, the increase of R-RAA aPL in MCI patients is demonstrated by using redox-reactive iron in the form of hemin.

It is possible that such an *in vivo* increase represents a compensatory effect brought about by the conditions of oxidative stress in the MCI individual which causes unmasking and consumption of the R-RAA thereby promoting additional R-RAA production.

In conclusion, our results strongly support R-RAA aPL measurement as a promising serum diagnostic biomarker for the early diagnosis of AD. Further collaborative studies planned with the ADNI will be designed to assess aspects of selectivity of the biomarker for AD v. other neurological diseases. We will also conduct studies to assess both inter and intra patient variability, and diurnal fluctuations of the biomarker levels in serum in a larger population. These studies will be necessary to begin to build a database from which the relative predictability of the biomarker for staging AD following a single determination can be assessed. Advanced studies are envisaged with serum samples collected longitudinally from a larger group of individuals whose AD disease status is determined at autopsy. These data will be used to determine whether the sensitivity for detection of early AD is enhanced by following the changes in the R-RAA aPL biomarker over time in a given individual. This will establish feasibility for developing the R-RAA aPL diagnostic as a routine screening technology for individuals at-risk for developing AD. As with any new biomarker diagnostic that has the potential for identifying individuals at risk for developing a life-threatening disabling diseases, acceptance will only come after the diagnostic yields proven accurate and predictive results when used in a large population of individuals over time.

References

1. *Li, S. D., Su, Y. D., Li, M., and Zou, C. G. (2006) Hemin-mediated hemolysis in erythrocytes: effects of ascorbic acid and glutathione, Acta biochimica et biophysica Sinica 38, 63-69.*
2. *McIntyre, J. A., Wagenknecht, D. R., and Ramsey, C. J. (2009) Redox-reactive antiphospholipid antibody differences between serum from Alzheimer's patients and age-matched controls, Autoimmunity, 42, 646-652.*
3. *Agar, C., van Os, G. M., Morgelin, M., Sprenger, R. R., Marquart, J. A., Urbanus, R. T., Derksen, R. H., Meijers, J. C., and de Groot, P. G. (2010) Beta2-glycoprotein I can exist*

in 2 conformations: implications for our understanding of the antiphospholipid syndrome, Blood 116, 1336-1343.

4. *Sugi, T., and McIntyre, J. A. (1995) Autoantibodies to phosphatidylethanolamine (PE) recognize a kininogen-PE complex, Blood 86, 3083-3089.*
5. *McIntyre, J. A., Wagenknecht, D. R., and Sugi, T. (1997) Phospholipid binding plasma proteins required for antiphospholipid antibody detection--an overview, Am J Reprod Immunol 37, 101-110.*
6. *McIntyre, J. A., Wagenknecht, D. R., and Ramsey, C. J. (2009) Redox-reactive antiphospholipid antibody differences among ADNI provided serum samples from Alzheimer's patients,mild cognitive impaired patients and controls, (unpublished observations).*
7. *DeKosky, S. T., Ikonomovic, M. D., Styren, S. D., Beckett, L., Wisniewski, S., Bennett, D. A., Cochran, E. J., Kordower, J. H., and Mufson, E. J. (2002) Upregulation of choline acetyltransferase activity in hippocampus and frontal cortex of elderly subjects with mild cognitive impairment, Ann Neurol 51, 145-155.*
8. *Bruce-Keller, A. J., Gupta, S., Parrino, T. E., Knight, A. G., Ebenezer, P. J., Weidner, A. M., LeVine, H., 3rd, Keller, J. N., and Markesbery, W. R. (2010) NOX activity is increased in mild cognitive impairment, Antioxid Redox Signal 12, 1371-1382.*
9. *Smith, M. A., Zhu, X., Tabaton, M., Liu, G., McKeel, D. W., Jr., Cohen, M. L., Wang, X., Siedlak, S. L., Dwyer, B. E., Hayashi, T., Nakamura, M., Nunomura, A., and Perry, G. (2010) Increased iron and free radical generation in preclinical Alzheimer disease and mild cognitive impairment, J Alzheimers Dis 19, 363-372.*
10. *Padurariu, M., Ciobica, A., Hritcu, L., Stoica, B., Bild, W., and Stefanescu, C. (2010) Changes of some oxidative stress markers in the serum of patients with mild cognitive impairment and Alzheimer's disease, Neurosci Lett 469, 6-10.*
11. *Lavados, M., Guillon, M., Mujica, M. C., Rojo, L. E., Fuentes, P., and Maccioni, R. B. (2008) Mild cognitive impairment and Alzheimer patients display different levels of redox-active CSF iron, J Alzheimers Dis 13, 225-232.*

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