

## Hemoglobin binding to A $\beta$ and *HBG2* SNP association suggest a role in Alzheimer's disease

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### Abstract

From a normal human brain phage display library screen we identified the gamma (A)-globin chain of fetal hemoglobin (Hb F) as a protein that bound strongly to A $\beta$ 1-42. We showed the oxidized form of adult Hb (metHb A) binds with greater affinity to A $\beta$ 1-42 than metHb F. MetHb is more toxic than oxyhemoglobin because it loses its heme group more readily. Free Hb and heme readily damage vascular endothelial cells similar to Alzheimer's disease (AD) vascular pathology. The *XmnI* polymorphism (C  $\rightarrow$  T) at -158 of the gamma (G)-globin promoter region can contribute to increased Hb F expression. Using family-based association testing, we found a significant protective association of this polymorphism in the NIMH sibling dataset ( $n = 489$ ) in families, with at least two affected and one unaffected sibling ( $p = 0.006$ ), with an age of onset  $>50$  years ( $p = 0.010$ ) and  $>65$  years ( $p = 0.013$ ), and families not homozygous for the *APOE4* allele ( $p = 0.041$ ). We hypothesize that Hb F may be less toxic than adult Hb in its interaction with A $\beta$  and may protect against the development of AD.

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### 1. Introduction

#### 1.1. A $\beta$ and cerebral amyloid angiopathy

Alzheimer's disease (AD), a neurodegenerative disorder with a complex etiology and pathogenesis, is characterized by progressive loss of memory and cognitive functions. Beta amyloid peptide (A $\beta$ ) appears to be central to the pathogenesis of AD. Derived from the amyloid protein precursor, A $\beta$  aggregates in plaques in the brain and in cerebral vessels are a diagnostic feature of AD (Hardy and Selkoe, 2002). A $\beta$  deposited in plaques in the walls of

cerebral blood vessels causes cerebral amyloid angiopathy (CAA) (Vinters and Vonsattel, 2000), and is available to bind to circulating cells including erythrocytes (Miao et al., 2005; Nicoll et al., 2004; Ravi et al., 2005). Amyloid deposits in cerebral vessels can obliterate the lumens of cerebral arteries and damage the endothelium and basal lamina, causing a breakdown of the blood brain barrier (BBB), ischemia, and neurodegeneration (Krizanac-Bengez et al., 2004; Nicoll et al., 2004; Wisniewski et al., 2000). Interestingly, vascular changes, including hypoperfusion, appear to precede evidence of neuronal injury (de La Torre, 2002). Morphological and inflammatory changes are consistently observed in the brain vasculature in AD (de La Torre, 2002; Suo et al., 1998).

### 1.2. A $\beta$ binding with Hb

Proteins that interact with A $\beta$  may activate or enhance its deposition or pathogenicity, and thus contribute to the development of AD. While isolating specific proteins with strong affinity for A $\beta$ , one group of investigators reported the  $\alpha$  chain of hemoglobin (Hb) as one of only a few proteins recovered and identified from rat brain homogenates (Oyama et al., 2000). In addition, Hb has been co-immunoprecipitated with A $\beta$  from both AD brains and plasma (Kuo et al., 2000; Oyama et al., 2000; Wu et al., 2004).

### 1.3. Hb and heme in AD brains and its vasculature

Abnormal levels of Hb and heme have been associated with brain and vascular tissue in AD. Hb, Hb derived peptides, and Hb mRNA levels have been reported to be increased in AD brains relative to the brains of non-demented control subjects (Cullen et al., 2005; Poljak et al., 2004; Schonberger et al., 2001; Slemmon et al., 1994; Wu et al., 2004). Brain Hb levels in AD were highest in the hippocampus and parietal gray and white matter and lowest in the cerebellum, and there was co-localization of Hb with senile plaques and CAA (Wu et al., 2004). The increased presence of Hb and its breakdown products in the brain is probably derived from erythrocytes in the circulation as a result of injury to the endothelium and the BBB with subsequent leakage of plasma or blood components into the perivascular space where additional heme iron-mediated damage may occur (Cullen et al., 2005).

### 1.4. A $\beta$ damage to erythrocytes

Circulating blood cells are exposed to both soluble A $\beta$ 1-40/42 and to A $\beta$  aggregates associated with the luminal surfaces of cerebral microvessels (e.g. CAA) (Ravi et al., 2005; Rogers et al., 2005). *In vitro*, A $\beta$ 25-35 induces rapid lysis of human erythrocytes, whereas A $\beta$ 1-42 induces delayed lysis of erythrocytes that can be attenuated by antioxidants (Mattson et al., 1997). Human red blood cells (RBCs) that bind fibrillar amyloid *in vitro* and *in vivo* show increased RBC hemoglobin oxidative modification and endothelial adhesion (Galeazzi et al., 2002; Jayakumar et al., 2003; Ravi

et al., 2005; Rogers et al., 2005). AD patients have increased RBC membrane injury suggesting the increased potential for erythrocyte lysis and liberation of Hb when the cells are exposed to A $\beta$ 1-42 (Bosman, 1991; Goodall et al., 1994; Mattson et al., 1997; Solerte et al., 2000).

### 1.5. Oxidized heme and damage to vascular cells

Extracellular or free Hb released from lysed erythrocytes causes injury to endothelial cells (Balla et al., 1993; Liu and Spolarics, 2003; Rother et al., 2005) and death of cultured neurons (Everse and Hsia, 1997; Regan and Panter, 1996; Sadrzadeh et al., 1987). Hb contained in RBCs is normally retained in the reduced state (oxyHb; Fe<sup>2+</sup>). Free Hb undergoes spontaneous oxidation to methemoglobin (metHb, Fe<sup>3+</sup>) which loses its heme group more readily than oxyHb (Everse and Hsia, 1997; Minneci et al., 2005). Oxidized heme is a pro-oxidant that damages vascular cells, where the iron derived from heme becomes available to produce a variety of reactive oxygen species via the Fenton reaction (Juckett et al., 1998; Wagner et al., 2003) resulting in membrane lipid peroxidation and damage to DNA and proteins (Howlett et al., 1997; Kalaria, 1997; Markesbery, 1999). These actions could account for some of the vascular pathology and neuronal injury or death in AD. Additionally, competition for freed Hb, outside of the normal haptoglobin and related scavenging systems, may permit or enhance vascular injury.

We present here our results of a phage display screen of a human brain cDNA library to identify proteins that interact with A $\beta$ , and the gamma (A)-globin subunit of fetal hemoglobin (Hb F) was identified. We also present surface plasmon resonance studies that show differential binding of adult Hb (Hb A) and Hb F, in oxidized and reduced states, to A $\beta$ 1-42. Specifically, metHb F showed reduced affinity for binding to A $\beta$  relative to metHb A. Hb F contains two gamma chains (either A or G) in place of the two beta chains of Hb A (Miller, 2005). Ten to 35% of persons in the general population have a common promoter polymorphism, C  $\rightarrow$  T<sup>-158</sup> *XmnI*, in the gene coding for the gamma (G)-globin chain, *HBB2*, that can contribute to increased levels of the usually small amount of Hb F present in normal individuals and further increases in levels when they are under hematological stress (Garner et al., 2005; Gilman, 1988; Ho et al., 1998; Leonova et al., 1996; Wood, 2001). We genotyped the *XmnI* polymorphism in the NIMH AD cohort and present the results here. We discuss the implications of these results and how Hb may play a role in the pathogenesis of AD.

## 2. Methods

### 2.1. Phage display screening for amyloid-binding proteins

A phage display of a human brain cDNA library (#K1006-2 & #HL6001XA, Clontech, Mountain View, CA) was used

to screen for binding partners for aggregated A $\beta$ 1-42 following the manufacturers' directions (user manual Clontech PT3084-1). The phage were biopanned against immobilized A $\beta$ 1-42 (#H-1368, Bachem, Torrance, CA). A $\beta$ 1-42 was dissolved in water (10  $\mu$ g/ml) and 100  $\mu$ l was added to each well of a 96-well high-capacity protein binding plate (#9502-920-00P, Labsystems, Franklin, MA). Binding and aggregation of A $\beta$ 1-42 in 96-well plates and preparation of negative control plates follow general methods (Behl et al., 1994; Burdick et al., 1992; Kay et al., 1996). A negative control plate was baited using a scrambled A $\beta$ 25-35 sequence (KSGNMLGI-IAAG) (Behl et al., 1994). After three rounds of biopanning, a subset of 87 individual clones from the final enriched phage library pan were screened for binding to immobilized A $\beta$ 1-42 and scrambled A $\beta$ 25-35. The amount of specifically bound phage was assessed by detecting pVIII coat protein in an ELISA format. Clones exhibiting absorbance ratios  $\geq 2.5$  were processed for plasmid DNA isolation using Wizard Plus SV Minipreps (#A1330; Promega Corporation, Madison, WI). Isolated plasmids were sequenced in the Medical College of Georgia Molecular Biology Core Facility using the dye terminator method (ABI Prism Model 377) and the 5'-sequencing primer provided with the phage library.

## 2.2. Surface plasmon resonance protein–protein interaction assay

A BIACORE X instrument (Piscataway, NJ) with two microfluidic flow cells (fc) was used for analysis of unlabeled protein–protein interactions. Amyloid was allowed to aggregate and buffers were prepared as previously described (Shuvaev and Siest, 1996). Control analytes and the experimental analytes were evaluated for interaction with immobilized A $\beta$ 1-42. Each analyte was injected at a concentration of 1  $\mu$ M in running/sample buffer (10 mM Na<sub>2</sub>PO<sub>4</sub>, 150 mM NaCl, pH 7.4) across the fc1 (control) and fc2 (A $\beta$ 1-42) surfaces at a flow rate of 20  $\mu$ l/min for 3 min, then buffer alone was flowed over the surfaces for at least an additional minute to determine the disassociation kinetics. Following each analyte injection, the control and active flow cells are regenerated with 4 M Gdn HCl (10 mM Tris–HCl, pH 8.0). Binding data are corrected by subtracting the response observed in fc1 (control) from that observed in fc2 (A $\beta$ 1-42). Dissociation equilibrium constants and rate constants were determined by fitting corrected data to a 1:1 Langmuir binding model using global analysis (BIAevaluation 3.0 Software) and reported data was repeated at least twice.

For flow cell surface preparation, aggregated A $\beta$ 1-42 (#H-1368, Bachem) was immobilized in fc2 of a CM5 sensor chip by amine coupling according to the manufacturer's guidelines (amine coupling kit, Biacore Inc.). Following activation of the carboxymethylated dextran surface, 35  $\mu$ l of A $\beta$ 1-42 (100 g/ml in 10 mM CH<sub>3</sub>COONa, pH 4.0) was injected across the activated surface at a flow rate of 5  $\mu$ l/min. Following A $\beta$ 1-42 immobilization, unreacted surface groups were blocked as usual. Non-covalently bound A $\beta$ 1-42 was

removed by multiple injections (20  $\mu$ l at 20  $\mu$ l/min) of regeneration buffer (4 M Gdn HCl). A stable A $\beta$ 1-42 baseline of 1700 response units (RUs; 1000 RUs = 1 ng protein/mm<sup>2</sup>) was achieved following four regeneration injections.

Control analytes consisted of positive [Apolipoprotein E4 (APOE4) (#178476, Calbiochem/EMD Biosciences, San Diego, CA); anti-amyloid antibody (mouse IgG1 monoclonal antibody to beta-amyloid, #M0872, DAKO, Carpinteria, CA)] and negative controls [bovine serum albumin (BSA, #A7638, Sigma, St. Louis, MO); anti-neurofilament antibody (mouse IgG1 monoclonal antibody to phosphorylated NFH & NFM, #SMI-31, Sternberger Monoclonals Inc., Lutherville, MD)]. The same anti-beta amyloid antibody was used to determine if the amyloid peptide surface was still intact following multiple analyte and regeneration runs.

Experimental analytes consisted of adult methemoglobin (metHb A), adult oxyhemoglobin (oxyHb A), fetal methemoglobin (metHb F), fetal oxyhemoglobin (oxyHb F), and gamma (G)-globin chains. Hbs were obtained by the Medical College of Georgia Sickle Cell Center from blood samples donated by patient volunteers using IRB approved informed consent forms. Hbs F were obtained from a beta thalassemia patient (>90% Hb F) or newborns using left over blood samples acquired for clinical tests under IRB approval; no patient identifiers were associated with clinical samples. MetHb was derived from fresh oxyHb that was oxidized by exposure to air at 4 °C for several weeks and assayed by cooximetry as being over 95% metHb. Hbs were liberated from washed (Locke' solution, 154 mM NaCl, 5.6 mM KCl, 2.3 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 3.6 mM NaHCO<sub>3</sub>, 5 mM D-glucose) RBC by exposure to 4 $\times$  volumes of distilled H<sub>2</sub>O. Cell debris was removed by centrifugation (12,000  $\times$  g/10 min) and the supernatant was used. In some cases commercially available adult human metHb was also used (#H7379, Sigma). Individual globin chains were derived by HPLC separation as previously described (Kutlar and Huisman, 1993).

## 2.3. Study population: NIMH AD genetic initiative families

The family study group comprised 209 families with at least two siblings with AD with mean age of onset (MAO) 70.9  $\pm$  7.4 (range 50–97 years) years and one unaffected sibling (used as a control). This group (candidate gene set) is a subset of a larger group of families collected as part of the NIMH AD genetics initiative (Blacker et al., 2003) following a standardized protocol utilizing the NINCDS-ADRDA criteria for diagnosis of definite and probable AD (Tierney et al., 1988). Because parental genotypes are usually not available for this dataset, family-based association testing (FBAT) was used (Lake et al., 2000). The candidate gene set containing 203 families with age of onset  $\geq 50$  years (non-early families) was further subdivided into two subsets by age of onset: 61 non-late onset families (age of onset between 50 and 65 years) and 142 late onset families (age of onset > 65 years). Six families with ages of onset under 50 that were part of

the total set were excluded from this set (non-early). This set of 203 non-early families was also classified by *APOE4* dosage, wherein 64 families were designated as *APOE4E4* member (at least one member of the family is homozygous for the E4 allele) and 139 families classified as no member *E4E4* (no individuals in the family possessing the *E4E4* genotype). Individuals who were heterozygous for the *APOE3/E4* genotype are included in families in both of these subsets. Stratifying on homozygous *APOE4* status rather than carrier status separates families with strong *APOE4* presence from those with no or less *APOE4* presence.

#### 2.4. *XmnI* genotyping of $-158$ polymorphism of *HBG2* in the NIMH AD cohort

Isolation of genomic DNA and general procedures for PCR-RFLP genotyping have been described (Perry et al., 2001). Reagents and conditions to amplify the *XmnI* polymorphism (C  $\rightarrow$  T) at bp  $-158$  of *HBG2* were, 0.5 pmol each of left (5'-GCACTGAACTGTTGCTTTATAGGAT-3') and right primers (5'-GCGTCTGGACTAGGAGCTTATTG-3'), 0.5 U of Taq (Promega, Madison, WI) and 37 cycles of 94 °C/40 s, 55 °C/30 s, 72 °C/1 min with a final extension of 72 °C/8 min. After cycling, temperature was lowered to 12 °C/30 min to reduce condensation on the microtiter plates. Three microliters of product was digested with 2 U of *XmnI* (N. E. Biolabs, Beverly, MA) in a 12  $\mu$ l reaction at 37 °C/4 h. Digestion products were run on 1.5% agarose gel, and photographed on a Fluor-S Imager (Bio-Rad, Hercules, CA).

### 3. Results

#### 3.1. Amyloid-binding proteins isolated from phage display clones

The absorbance ratios ( $A\beta$ 1-42 well/scrambled  $A\beta$ 25-35 well) ranged from 0.85 to 3.14 absorbance units. We selected a subset of clones that bound to  $A\beta$ 1-42 coated wells, but not to scrambled  $A\beta$ 25-35 coated wells and with absorbance ratios greater than 2.5. These clones were isolated and sequenced. Three clones had novel sequences bearing receptor-like motifs (data not shown). One of the positive clones corresponded to a fragment (Leu81–Val137) of the gamma (A)-globin subunit of Hb F; this peptide sequence included the heme binding site.

#### 3.2. Surface plasmon resonance of $A\beta$ 1-42 binding

Since there is evidence that the  $A\beta$  peptide can exert its neurotoxic effects via iron-mediated oxidative damage (Rottkamp et al., 2001; Schubert and Chevion, 1995; Schubert et al., 1995), we further investigated and characterized the interaction and binding properties of  $A\beta$  to hemoglobin, which contains bound iron, by performing surface plasmon analysis. Fig. 1 shows that the amyloid surface

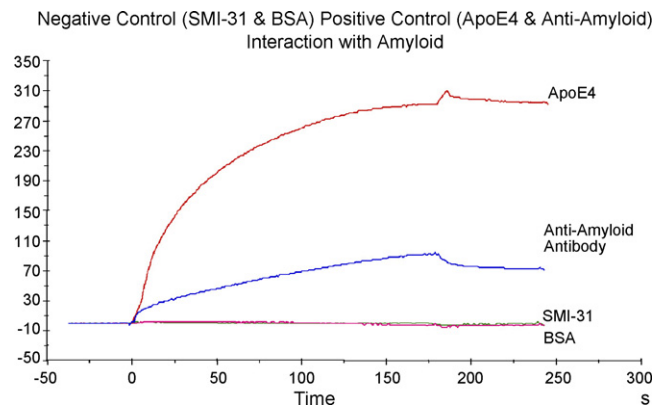


Fig. 1. Positive controls (1 M *APOE4* and 7 nM anti-amyloid monoclonal antibody) and negative controls (1 M monoclonal antibody SMI-31 and 1 M BSA). Data shown is corrected for bulk effects by subtracting the response observed in fc1 from that observed in fc2.

in fc2 binds the positive control analytes, *APOE4* and the amyloid antibody, but does not bind the negative controls, BSA or a non-specific antibody (SMI-31) of the same isotype (IgG1). Analysis of the surface resonance data from the Hb analytes (Fig. 2) fit a 1:1 Langmuir binding model (BIAevaluation 3.0) using the kinetic parameters listed in Table 1. This suggests that the globin chain, without an attached heme group, and both the adult and fetal metHbs bind to  $A\beta$ 1-42 with a single on and off rate, indicating that there is a single binding site on the analytes and the ligand. Table 1 also shows the gamma (G)-globin chain and the metHbs have relatively fast and roughly equivalent on rates with slow off rates. However, the metHbs, particularly metHb A, have slower off rates relative to the naked globin chain. In contrast, adult or fetal oxyHbs exhibit no significant binding to  $A\beta$ 1-42.

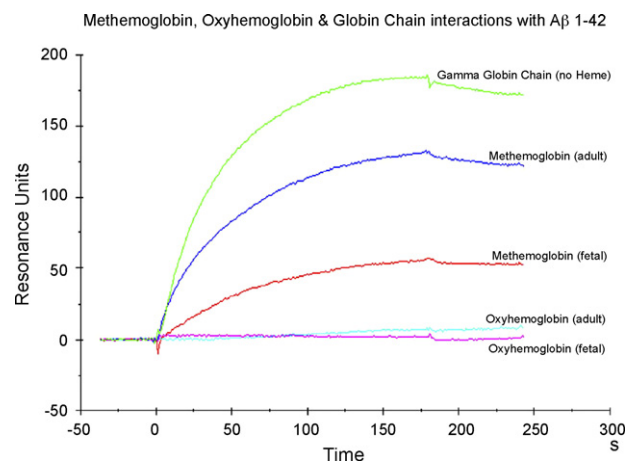


Fig. 2. Data shown is corrected for bulk effects by subtracting the response observed in fc1 from that observed in fc2. Binding of the amyloid peptide ( $A\beta$ 1-42) appears to be mediated by the redox state of the heme iron. Globin without a heme group or hemoglobin where the heme iron is oxidized to  $Fe^{3+}$  (metHb) is permissive for binding. In contrast, when the heme iron is in the  $Fe^{2+}$  reduced state (oxyHb), binding is blocked.

Table 1  
Surface plasmon resonance kinetic analysis: fit to a 1:1 Langmuir binding model

Analyte	$k_a$ (M $s^{-1}$ )	$k_d$ (s $^{-1}$ )	$K_D$ (M)	$R_{max}$ (RU)	$\chi^2$
Gamma (G)-globin	21,900 ± 99	1.13e-3 ± 3.58e-5	5.19e-8	196 ± 0.551	1.38
MetHb A	17,600 ± 144	2.20e-7 ± 5.22e-8	1.25e-11	130 ± 0.197	1.38
MetHb F	12,900 ± 237	5.76e-4 ± 8.30e-5	4.47e-8	63.4 ± 0.722	1.38
OxyHb A	NF	NF	NF	NF	NF
OxyHb F	NF	NF	NF	NF	NF

Kinetic data from Fig. 2: Standard errors are located below the values. An association rate constant,  $k_a$ , of 2e4 means that 20,000 binding events occur per second when the concentration of the analyte is 1 M. A dissociation rate constant,  $k_d$ , of 1e-3 means that 0.001 complexes fall apart per second when the concentration of the analyte is 1 M. NF = does not fit the 1:1 Langmuir model.  $R_{max}$  is maximum response unit (RU).

### 3.3. *XmnI* polymorphism of the gamma (G)-globin gene (*HBG2*)

Results of the family based association analyses (FBAT) (Lake et al., 2000) for the *XmnI* polymorphism (C → T) at bp -158 of *HBG2* in the candidate gene set of DNA samples and the other subsets are shown in Table 2. There was a significant negative association of the *XmnI* promoter polymorphism with the presence of AD in the total candidate gene set and the non-early (age of onset > 50 years), late onset (age of onset > 65 years), and no member E4E4 (no individuals in the family possessing an E4E4 genotype) subsets. The T allele of the *XmnI* promoter polymorphism was transmitted less frequently to patients with AD than the normal (C) allele.

The association appears to be more prominent in the late-onset families than in the early onset families, which is reflected in its association in the no member E4E4 subset. The latter subset has a higher age of onset because the E4 allele is associated with earlier onset of disease (Lange and Laird, 2002). When power analysis was performed, an effect size which resulted in 80% power in the larger groups (total, late, no member E4E4), yielded < 50% power in the smaller subsets (non-late, E4E4 member). Therefore, the non-significant results for these latter subsets could be due to insufficient power.

Table 2  
*p*-Values for -158 (C → T) polymorphism of *HBG2*

Subset of DNA	Z statistic	<i>p</i> -Value (dominant model)
Total candidate gene set (209 families)	-2.773	0.006*
Age of onset ≥ 50 subset (203 families)	-2.570	0.010*
Age of onset 50–65 subset (61 families)	-0.865	0.387
Age if onset > 65 subset (142 families)	-2.471	0.013*
<i>APOE4E4</i> member subset (64 families)	-1.454	0.146
No E4E4 member subset (139 families)	-2.045	0.041*

Z statistic for T allele: A positive value of Z statistic indicates more transmission and a negative value is less transmission to affected individuals than expected under the null hypothesis.

\* Indicates significantly associated with AD.

## 4. Discussion

From a phage display screen of a human brain cDNA library seeking A $\beta$ -binding proteins, we identified the gamma (A)-globin subunit of fetal hemoglobin as a binding partner. In that only a sub-population of clones in the phage display were sequenced and identified, we did not determine if other globin chains besides the gamma (A) chain also bound to A $\beta$  using this approach. However, the coding regions of *HBG1* and *HBG2*, that code for the gamma (A) and gamma (G)-globin chains of Hb F, respectively, exhibit a high degree of homology differing only by a single base pair (Schroeder et al., 1968). Therefore, it is likely that the gamma (G)-globin chain binds A $\beta$  with the same high avidity as the gamma (A) chain. Furthermore, additional A $\beta$  binding proteins, such as APOE, APOJ, and alpha 2 macroglobulin, could have been present in clones not selected and sequenced by us.

Our surface plasmon resonance studies confirmed that both Hb A and Hb F specifically bind to A $\beta$ 1-42. However, binding occurs only when the iron in the heme group is in the oxidized state (metHb), and that metHb A binds to A $\beta$  with significantly higher affinity than metHb F (Table 1). Furthermore, the globin chains lacking the heme prosthetic group avidly bind amyloid peptide. This suggests that the binding of A $\beta$  peptides to Hb is primarily via the globin polypeptide, rather than directly to the heme group. These studies also demonstrate that the binding of the globin chain and metHbs to A $\beta$  are in the range of efficient ligand-receptor binding, although somewhat lower than the known A $\beta$  binding protein, APOE4, which binds strongly to A $\beta$ .

There is *in vivo* and *in vitro* evidence of RBCs binding A $\beta$  and of A $\beta$  mediated RBC injury (Galeazzi et al., 2002; Giunta et al., 2005; Jayakumar et al., 2003; Mattson et al., 1997; Ravi et al., 2005). Indeed, over 50% of A $\beta$  in circulation within the vascular compartment may be bound to RBCs (Rogers et al., 2005). It is clear that RBCs in AD show morphological and biochemical evidence of injury that can lead to hemolysis (De Franceschi et al., 2004; Engstrom et al., 1995; Gibson and Huang, 2002; Janoshazi et al., 2006; Kawamoto et al., 2005; Ravi et al., 2005; Repetto et al., 1999), although it is not clear if hemolytic anemia is associated with AD (Beard et al., 1997; Fujiwara et al., 2003; McCaddon et al., 2004; Milward et al., 1999; Pandav et al., 2004). Additionally, while it is not known if the Hb isoform content (e.g. adult versus fetal) of

RBCs plays a role in RBC lysis, RBC bound A $\beta$  can cause oxidative injury to Hb within the RBC (Jayakumar et al., 2003) which may effect the stabilization of the heme group mediating its release and potential for endothelial injury. Free Hb may not only directly injure the vasculature, including endothelial cells, but may mediate blood flow through NO scavenging, resulting in hypoperfusion to the tissue (Azarov et al., 2005; Gladwin et al., 2004; Jeney et al., 2002; Liu and Spolarics, 2003). Indeed, hypoperfusion may be an early pathogenic event in AD (Hirao et al., 2005; Johnson et al., 1998; Spilt et al., 2005).

The haptoglobin/hemopexin system normally binds extracellular Hb and heme (Anderson and Frazer, 2005). However, the protective haptoglobin/hemopexin/albumin/heme oxygenase (HO) systems can be overwhelmed in disease states, including hemolysis and inflammation, resulting in increased circulating levels of free Hb and free (or LDL bound) heme that can attack the endothelium and effect perfusion (Balla et al., 2005; Jeney et al., 2002; Minneci et al., 2005; Na et al., 2005; Rother et al., 2005; Tabbara, 1992). Such may be the case in AD when RBC fragility and lysis are increased. Increased free Hb in human serum has recently been found associated with AD relative to controls (Zhang et al., 2004). This and several other reports indicate that haptoglobin is increased in AD plasma and CSF (Johnson et al., 1992; Mattila et al., 1994; Yu et al., 2003). This may represent an attempt to adjust the homeostatic level in response to chronic hemolysis, although inflammation, which is also associated with AD, can lead to increased haptoglobin levels (Kormoczi et al., 2006). Indeed, a growing list of clinical manifestations (including hemolysis-associated smooth muscle dystonia, vasculopathy, and endothelial dysfunction) are being attributed to hemoglobin release, suggesting hemolysis and hemoglobinemia should be considered as a novel mechanism in various disease states (Balla et al., 2005; Kumar and Bandyopadhyay, 2005; Rother et al., 2005; Tabbara, 1992).

As mentioned above, free oxyHb spontaneously oxidizes to form metHb outside of the RBC and, in general, metHb is more toxic than oxyHb because it loses its heme group more readily (Everse and Hsia, 1997; Minneci et al., 2005). Heme released from metHb damages vascular endothelial cells by catalyzing oxidative injury and lipid peroxidation (Chiu and Liu, 1997; Lin et al., 2001; Liu and Spolarics, 2003; Szebeni et al., 1984; Yeh and Alayash, 2004). The strong affinity of metHb A for binding A $\beta$  might accelerate the normal “spontaneous” heme release when in the presence of the amyloid peptide or A $\beta$  may preferably enhance the injury and lysis of Hb A containing RBCs versus Hb F containing RBCs. In addition, amyloid may interact with free heme groups or generate free radicals in the presence of iron with its tighter association to metHb A (Howlett et al., 1997; Kalaria, 1997; Markesbery, 1999). Recent evidence indicates the A $\beta$ -heme complex acts as a peroxidase, resulting in increased oxidative damage (Atamna and Boyle, 2006).

The above observations are consistent with some of the morphological and inflammatory changes associated with the

vasculature in AD (De Jong et al., 1997; de La Torre, 2002, 2005; Roher et al., 2006; Suo et al., 1998). This may be, in part mediated by A $\beta$  interactions with RBCs and free Hb, and, in particular, with metHb A.

Hb F expression, restricted to a subset of erythrocytes called F Cells, varies with genetic and environmental factors (Boyer et al., 1975; Garner et al., 2002). It is estimated that 89% of Hb F and F cell variance in adults is due to heritable factors (Garner et al., 2000b) and the *XmnI* polymorphism (C  $\rightarrow$  T) at position  $-158$  of the *HBG2* promoter accounts for the greatest single share of this genetic variability (Garner et al., 2000a; Steinberg, 2005). High F cell and Hb F levels in normal populations can be due, at least in part, to the *XmnI* polymorphism (Garner et al., 2005; Leonova et al., 1996; Sampietro et al., 1992; Shimizu et al., 1992; Wood, 1993; Zertal-Zidani et al., 2002). Additionally, with hematological stress (e.g. sickle cell disease, beta thalassemia, hemolysis, pregnancy), the *XmnI* polymorphism has been shown to be at least partly responsible for an even greater increase in total Hb F and F cell levels (Adekile et al., 2002; Bandyopadhyay et al., 2005; De Angioletti et al., 2004; Ferrara et al., 2003; Gilman, 1988; Lolis et al., 1995; Miller et al., 1987; Steinberg, 2005; Thein et al., 1987).

The decreased transmission of the T allele in the AD patients relative to the unaffecteds that we found may indicate that the presence of the T allele provides a protective mechanism due to the higher Hb F:Hb A ratio. Because metHb F does not bind A $\beta$ 1-42 as avidly as metHb A, the interaction of metHb F with A $\beta$  may be less toxic than the interaction of metHb A with A $\beta$  resulting in less RBC lysis and damage to the vascular epithelium. Evidence for the contribution of the *XmnI* polymorphism in the protective role of Hb F against vascular damage has been observed in patients with sickle cell disease (Adekile et al., 2002; Miller et al., 1987; Steinberg, 2005). These results suggest that further investigation into the effects of the *XmnI* polymorphism upon Hb F levels in AD subjects may provide additional evidence supporting this hypothesis.

We also genotyped this polymorphism in a small cohort of African American AD patients ( $n = 126$ ) and age-matched controls ( $n = 93$ ) (data not shown). Although we did not show any statistically significant association, the direction of the outcomes was the same (C allele increased in AD patients). The lack of significance could be explained by small sample size and therefore, lack of statistical power.

In conclusion, we identified the gamma subunit of Hb F from the initial screening of a normal human brain phage display library while isolating proteins that bind to A $\beta$ 1-42. Using plasmon studies, we further investigated the kinetics of this interaction and found there was much stronger binding of oxidized or metHb (Fe<sup>3+</sup>) to A $\beta$ 1-42 when compared to reduced or oxy Hb (Fe<sup>2+</sup>), and that oxidized adult Hb (metHb A, 2 $\alpha$ 2 $\beta$  chains) binds with much greater affinity to A $\beta$ 1-42 than the oxidized form of fetal Hb (metHb F, 2 $\alpha$ 2 $\gamma$ ). Because the *XmnI* polymorphism in the promoter region of the gene, *HBG2*, which codes for the gamma (G)-globin sub-

unit of Hb F is the most common known genetic factor that contributes to adult Hb F expression, we genotyped this polymorphism in the NIMH cohort of AD patients and unaffected siblings. We observed a significant association to AD with a higher frequency of the ‘normal’ C allele of the *HBG2* polymorphism. In contrast, unaffected individuals showed greater transmission of the T allele, which has been reported to be associated with increased adult *HBG2* expression and Hb F levels. The increased percentage of Hb F in the circulating blood, if present, could be a mechanism that reduces damage to vascular epithelium due to the decreased binding affinity that metHb F has for A $\beta$ 1–42 in comparison to metHb A, or the reduced injury and lysis of Hb F containing RBCs (F cells) versus Hb A containing RBCs. Specifically, if there is increased Hb F due to the presence of the T allele, the change in the Hb A to Hb F ratio could result in: (1) decreased A $\beta$  interaction with Hb A resulting in less interference with the Hb scavenging systems and/or (2) reduced interaction of A $\beta$  with free metHb A resulting in reduced heme release and subsequent heme mediated injury; and/or (3) diminished A $\beta$  mediated lysis of F cells relative to Hb A containing RBCs resulting in reduced free Hb and heme and their toxic effects upon the vascular cells. Further, decreased RBC lysis and Hb/heme mediated damage to the microvasculature may help reduce hypoperfusion and inflammation in the brain as well. As such, results of this study suggest further investigations into the association of the *XmnI* polymorphism to AD, the effects of the *XmnI* polymorphism upon Hb F levels in AD subjects, and the role of differential binding of A $\beta$ 1–42 with Hb isoform content of RBC are needed.

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