Human microglial cells synthesize albumin in brain.

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Albumin has been implicated in Alzheimer's disease since it can bind to and transport amyloid beta, the causative agent; albumin is also a potent inhibitor of amyloid beta polymerization. In a pilot phase study of Human Brain Proteome Project, we found evidence that albumin may be synthesized in immortalized human microglial cells, human primary microglial cells, and human fetal and adult brain tissues. We also found the synthesis and secretion is enhanced upon microglial activation by Aβ_{1-42},

lipopolysaccharide treatment or human Alzheimer's brain.

Albumin is the most abundant plasma protein with multifunctional properties such as ligand-binding and transport, maintaining the colloid osmotic pressure of plasma, and regulating neutrophil function. Albumin is mainly synthesized in the liver at a rate of ~12 g per day, representing ~25% of total hepatic protein synthesis[2]. Albumin has been implicated in Alzheimer's disease (AD) since it can bind to and transport amyloid beta (A β)[3], the causative agent of AD; albumin is also a potent inhibitor of A β polymerization[4]. Despite the evidence of non-hepatic transcription of albumin in many tissues[5,6], non-hepatic synthesis of albumin at the protein level has been rarely confirmed. There has been no specific report about the synthesis of albumin in brain either at the mRNA level or at the protein level.

Albumin is not only a high-abundance protein in plasma, but also a major component of most extracellular fluids including interstitial fluid, lymph, and cerebrospinal fluid (CSF)[7-9] Albumin is found at a low concentration (~0.2 g/L) in CSF, yet it amounts to ~80% of the total CSF protein in contrast to ~60% as in plasma. CSF serum quotient of albumin, along with other blood-derived proteins in CSF, is widely used in the diagnosis of neurological diseases[10]. Albumin has been implicated in AD because it can specifically bind to and transport A β, the causative agent of AD, under physiological conditions. Moreover, albumin is a potent inhibitor of A β polymerization, representing ~60% of amyloid inhibitory activity in CSF and plasma[4].

To provide evidence of albumin synthesis in human microglial cells, three biospecimens were used: 1) immortalized human microglial cell line (HMO6); 2) human primary microglial cells; 3) human fetal and adult brain tissues. Additionally, Alzheimer's brain tissues were used to show the increased synthesis of albumin in microglial cells in AD, and all experimental procedures were described in **Supplementary Methods**.

We used immunostaining for albumin and microglial markers in human cells and brain tissues to confirm that albumin is expressed in microglial cells both *in vitro* and *in vivo*. In immunocytochemistry, all HMO6 cells were double-positive for microglial markers (CD11b and Iba1) and albumin (Fig. 1a); human primary microglial cells staining positive for Iba1 coexpressed albumin (Fig. 1b). In immunohistochemical study using human fetal and adult brain tissues, cells staining positive for microglial markers (CD11b and Iba1), also coexpressed albumin (Fig. 1c&d). When human adult brain tissues were double stained for albumin and non-microglial cell markers (i.e. microtubule-associated protein 2 (MAP2) for neurons; glial fibrillary acidic protein (GFAP) for astrocytes; myelin basic protein (MBP) for oligodendrocytes), minimal co-localization was observed (Fig. 1e-g).

To further confirm that albumin detected in HMO6 cells is not bovine albumin from the incubation media but human albumin, peptide sequencing using MS/MS was performed, which provided clear evidence that the albumin in HMO6 cells is human origin (Fig. 1h). The list of tryptic peptides identified using MS/MS is summarized in **Supplementary Table 1**.

Interestingly, the levels of albumin mRNA and protein increased upon microglial activation by A$\beta_{1\text{-}42}$ or lipopolysaccharide(LPS) treatments (Fig. 2). According to quantitative real-time PCR results, albumin gene transcription in HMO6 cells was more responsive to A$\beta_{1\text{-}42}$ than to LPS (Fig. 2a), which did not correlate well with albumin protein synthesis as illustrated by immunoblot analysis using HMO6 cell lysates (Fig. 2b). This may be partly explained by the fact that albumin is directly secreted from cells after synthesis. In accordance with this explanation, the level of albumin secreted by HMO6 cells into the incubation media also increased significantly after microglial activation, and was more responsive to A$\beta_{1\text{-}42}$ than LPS (Fig. 2c). In Alzheimer's brain, Iba1 and albumin were mostly co-localized (Fig. 2d).

In summary, we report here, for the first time, the synthesis of albumin in human microglial cells, the resident phagocytic cells in brain. Furthermore, our data show that the expression level of albumin in microglial cells increases upon activation with A$\beta_{1\text{-}42}$ or LPS treatments. These findings provide a new insight into the role of microglial cells in AD in which the 'amyloid hypothesis' that Aβ aggregates to trigger a complex pathologic cascade leading to neurodegeneration is generally accepted[11]. Microglial cells have been implicated in AD mainly because of their markedly elevated distribution in brain regions with Aβ deposition, and their pro-inflammatory functions[12]. Despite all the circumstantial evidence against microglial involvement in AD, it is still not clear whether microglial cells are 'friends or foes' in AD[13]. Our findings suggest that microglial cells may diminish A β deposition by increasing albumin synthesis and secretion. The capacity to bind to and transport A β enables albumin to inhibit A β polymerization and to increase A β clearance[14] .

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COMPETING INTERESTS STATEMENT

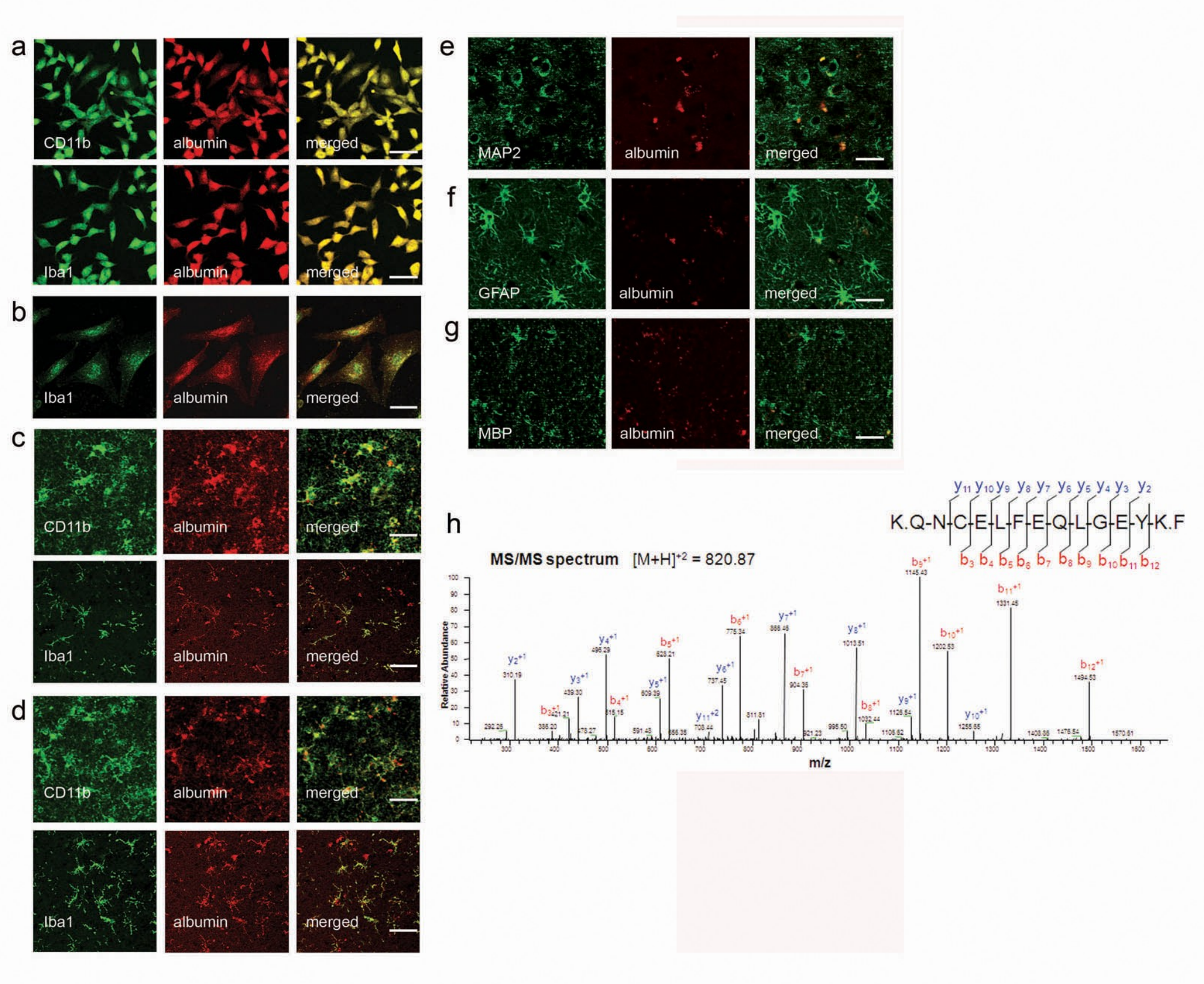
The authors declare no competing financial interests.

Figure 1. Albumin expression in human microglial cells and representative MS/MS spectra of a tryptic peptide of albumin. Microglial markers (CD11b or Iba-1) were co-expressed with albumin in all HMO6 cells (**a**) and human primary microglial cells (**b**). This observation was further confirmed by immunohistochemical staining of human fetal (**c**) and adult (**d**) brain tissues, in which albumin was co-expressed with CD11b or Iba-1. Minimal co-localization of albumin and non-microglial markers was observed in other cell types of brain: MAP2 for neurons (**e**); GFAP for astrocytes (**f**); MBP for oligodendrocytes (**g**). Scale bars = 50μm.

(**h**) Peptide sequencing provided clear evidence that albumin found in HMO6 cells is not bovine but human origin. The peptide QNCELFEQLGEYK is human-specific (i.e. the sequence is not 100% homologous with its bovine homologue, and thus distinguishable using MS/MS. The list of tryptic peptides identified using MS/MS is summarized in **Supplementary Table**.

Figure 2. Increase in albumin synthesis and secretion after microglial activation by A$\beta_{1\text{-}42}$, LPS treatments or human Alzheimer's brain tissue. qRT-PCR data (**a**) show that the transcription of albumin gene is significantly enhanced after microglial activation by A$\beta_{1\text{-}42}$ or LPS treatment. Immunoblot data also show that albumin synthesis increases at the protein level after microglial activation (**b**). In addition, immunoblot data show that anti-human-albumin antibody used does not have any cross-reactivity with bovine albumin in fetal bovine serum

(FBS), and that albumin is present in the incubation medium (**sup**, i.e. thus secreted from cells). ELISA data show that albumin secretion from HMO6 cells increased significantly after microglial activation (**c**). Moreover, the level of albumin in the incubation medium of A$\beta_{1\text{-}42}$ treated cells was significantly higher than that of LPS treated cells. **(d)** Microglial cells showed increased albumin staining intensity and size in human Alzheimer's brain tissue. Iba1, a microglial marker, and albumin were co-localized. Scale bar = 50µm.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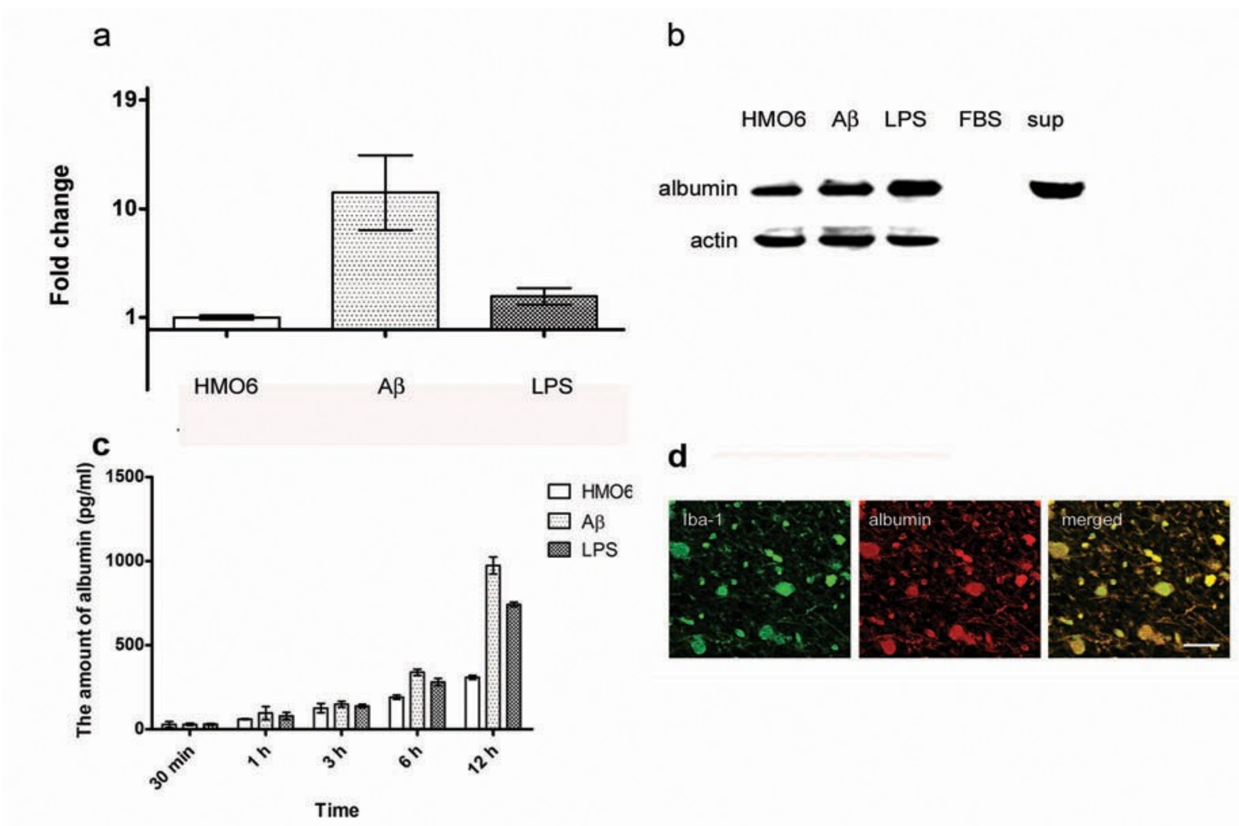
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SUPPLEMENTARY METHODS

Cell culture. For *in vitro* study, immortalized human microglial cell line (HMO6) was used. HMO6 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) with high glucose supplemented with 10% fetal bovine serum (FBS, Gibco) and 20 μg/ml gentamicin (Sigma), and incubated at 37 °C and 5% CO_2. Lipopolysaccharide (LPS) and A$\beta_{1\text{-}42}$ were acquired from Sigma-Aldrich and added to HMO6 cells at a concentration of 100 ng/mL and 5mM, respectively. Either LPS- or A$\beta_{1\text{-}42}$-treated HMO6 cells were harvested 6 hrs after treatment for further analysis except ELISA in which the incubation media were collected along 12 hrs time course for analysis.

Primary human microglial cell culture. Primary human microglial cells were prepared from embryonic human brains of 12-15 weeks gestation. The use of embryonic tissue samples were approved by Ethics Committee of the University of British Columbia, Faculty of Medicine. Briefly, brain tissues were incubated in phosphate-buffered saline (PBS) containing 0.25% trypsin and 40 µg/ml DNase I for 30 min at 37 °C. Dissociated cells were suspended in DMEM supplemented with 5% FBS, 5% horse serum, 20 µg/ml gentamicin, and 2.5 µg/ml amphotericine B (feeding medium), plated at a density of 10^6 cells/ml in 10 cm culture dish (10 ml), and incubated at 37 °C in an incubator with 5% CO_2/95% air atmosphere. After 2-3 weeks *in vitro*, microglia-enriched cultures were prepared by harvesting the floating cells in culture dish and replating them on Lab-Tek II Chamber Slide System (2 10^4 cells/wells, Nunc) for immunocytochemistry.

Immunoblot. Cell lysates were prepared with lysis buffer containing 7M urea, 2M thiourea, and 4% CHAPS. 2ml of the incubation medium was harvested, centrifuged at 1500 rpm for 10 minutes, and the supernatant was concentrated to about 15 µl using an Amicon centrifugal filter with 10-kDa nominal molecular weight limit (Millipore). Cell lysates, the incubation medium concentrate, and FBS were separated in 4-12% polyacrylamide gels (Invitrogen) and transferred to nitrocellulose membrane. The primary antibodies used were anti-human-albumin (1:1000, Abcam) that does not have cross-reactivity with bovine albumin, and anti-β-actin (1:1000, Cell Signaling).

Quantitative real-time PCR (qRT-PCR). RNA was isolated from six biological replicates from each group using Qiagen RNeasy MiniKit (Qiagen), pooled, and subjected to first-strand cDNA synthesis using Reverse Transcription System (Promega) according to the manufacturer's protocol. qRT-PCR was performed using Rotor-Gene 6000 (Corbett Lifescience), threshold cycle number and reaction efficiency were determined using Rotor-Gene 6000 series software version 2.7, and the $2^{-\Delta\Delta C_T}$ method was used for relative quantitation. The primers used were: 5'-ATGCCCCGGAACTCCTTTTC-3' (forward) and 5'-CAACAGGCAGGCAGCTTTAT-3' (reverse) for albumin, and 5'-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3' (forward) and 5'-TGACGGGGTCACCCACACACTGTGCCCATCTA -3' (reverse) for GAPDH.

Enzyme-Linked Immunosorbent Assay (ELISA). The amount of albumin in the cell incubation media was determined by human albumin BioAssay ELISA kit (US Biological). Six biological replicates were used, and each replicate was measured in duplicate.

Immunocytochemistry (ICC)

Cells were grown on Lab-Tek II chamber slide (Nunc), rinsed in PBS, fixed in 4% paraformaldehyde for 20 min, and rinsed again in PBS. The cells were incubated for overnight at 4 ∘C with mouse anti-human-albumin antibody (1:200, R&D system) that does not have cross-reactivity with bovine albumin, rabbit ant-Iba1 antibody (1:200, WAKO pure chemical industries) and rabbit ant-CD11b antibody (1:200, Abcam). The cells were rinsed in PBS and incubated for 1 hr at room temperature with tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-mouse IgG (1:500, Molecular Probes) and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (1:500, Molecular Probes). After wash in PBS, coverslips were mounted onto glass slides using Fluoroguard Antifade reagent (Bio-Rad Laboratories), and examined under a laser confocal fluorescence microscope (FV500, Olympus).

Immunohistochemistry (IHC)

IHC was performed as previously described. Human fetal (23wks), adult and Alzheimer's brain tissues were acquired from the Brain bank of Seoul National University hospital. The use of human brain tissues was approved by the institutional review board of Clinical Research Institute, Seoul National University hospital. Briefly, human adult, fetal brain tissues were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, followed by cryoprotection in 30% sucrose for overnight, and then 30 μm sections were prepared on a cryostat (Leica CM 1900). Paraffin-embedded 4 μm thick Alzheimer's brain tissue sections were de-paraffinized in xylene and rehydrated in a graded ethanol series. Antigen retrieval was performed by immersing slides in citrate buffer (pH 6) at 100 °C for 30 min. 10% normal goat serum was used to block non-specific binding. The tissue sections were incubated for overnight at 4 °C with mouse anti-albumin (1:100, R&D system), rabbit anti-Iba1 (1:200, WAKO), rabbit anti-CD11b (1:200, Abcam), rabbit anti-GFAP (1:200, Chemicon), rabbit anti-MAP2 (1:200, Chemicon) and rabbit anti-MBP (1:200, Chemicon). The tissues were rinsed in PBS and incubated for 1 hr at room temperature with TRITC-conjugated anti-mouse IgG (1:500, Molecular Probes) and FITC-conjugated anti-rabbit IgG (1:500, Molecular Probes). After wash in PBS, coverslips were mounted onto glass slides using Fluoroguard Antifade reagent (Bio-Rad Laboratories), and examined under a laser confocal fluorescence microscope (FV500, Olympus).

Immunoprecipitation of albumin

Cell lysates were prepared with RIPA buffer containing 1M Tris (pH 7.5), 5M NaCl, 10% NP-40, 10% deoxycholate and protease cocktail inhibitor. 1mg of cell lysates were incubated with 100 μl of anti-albumin Ab (Abcam)-conjugated Sepharose bead in 500 μl PBS at 4 °C for overnight. The Sepharose beads were precipitated at 14,000 rpm for 5 min, and washed with 1 ml of washing buffer containing 50 mM Tris–Cl and 500 mM NaCl (pH 8.0) for three times. The bound complexes were resolved on a 4-12% polyacrylamide gel (Invitrogen), and Coomassie-stained.

Protein sequencing using tandem mass spectrometry (MS/MS)

Gel bands were excised and subjected to in-gel digestion and MS/MS. All MS/MS experiments were performed using a Nano- LC/MS system consisting of a Surveyor HPLC system and a 7-tesla Finningan LTQ-FT mass spectrometer (Thermo Electron) equipped with a nano-ESI source. For peptide identification, MS/MS spectra were searched using Mascot version 2.0

(Matrix Science). Proteins that were identified by one or more high scoring peptides were considered to be true matches. The high scoring peptides corresponded to peptides that were above the threshold in our Mascot search (expected < 0.05, peptide score > 28). All high scoring MS/MS spectra were also manually validated.

Statistical analysis. Results are presented as mean ± s.e.m. Two-tailed Student *t*-tests were used for data analysis. A p-value of <0.05 was considered as the criteria of statistical significance.

Supplementary Table 1. Albumin peptides identified by MS/MS

Protein	Observed	Charge	Mr (expt)	Mr(calc)	Delta	Peptide ion Score	pepide sequence	Human specific*

	409.5405	3	1225.5996	1225.5978	0.0017	35.55	FKDLGEENFK	O
	575.3112	2	1148.6078	1148.6077	0.0001	62.83	LVNEVTEFAK	X
	717.7713	2	1433.528	1433.5261	1.0019	12.15	ETYGEMADCCAK	O
	566.5943	3	1696.7611	1696.7627	-0.0017	32.7	QEPERNECFLQHK	O
	464.2504	2	926.4862	926.4861	0.0001	43.7	YLYEIAR	X
	696.285	3	2085.8333	2085.8302	0.003	49.72	VHTECCHGDLLEC ADDR	O
	725.3246	2	1442.6379	1442.6347	-0.0001	67.89	YICENQDSISSK	O
ALB (IPI000 22434)	992.1225	3	2973.3457	2973.3371	0.0085	63.23	SHCIAEVENDEMP ADLPSLAADFVESK	O
	820.3983	2	1638.782	1638.7751	0.0068	67.62	DVFLGMFLYEYAR	O
	734.4257	2	1466.8373	1466.8357	0.0011	74.2	RHPDYSVVLLLR	O
	820.8709	2	1639.7273	1639.7188	0.0085	77.92	QNCELFEQLGEYK	O
	480.7849	2	959.5553	959.5552	0.0001	42.27	FQNALLVR	O
	547.3176	3	1638.9287	1638.9304	0.0004	89.2	KVPQVSTPTLVEVS R	X
	756.4253	2	1510.837	1510.8355	0.0006	76.78	VPQVSTPTLVEVSR	X
	500.8057	2	999.5983	999.5964	0.0004	57.11	QTALVELVK	X
	671.8216	2	1341.6303	1341.6274	0.0012	74.67	AVMDDFAAFVEK	O

Mr, molecular weight; expt, expected; calc, calculated.

*Human specific peptides (O) are not 100% homologous with bovine peptides, and thus distinguishable from their bovine homologues using MS/MS. Non-specific peptides (X) have 100% homology with bovine peptides.