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Small Heat Shock Proteins Induce a Cerebral Inflammatory Reaction

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More than 80% of Alzheimer's disease (AD) patients have some degree of cerebral amyloid angiopathy (CAA). In addition to arteries and veins, capillaries can also be affected. Capillary CAA (capCAA), rather than CAA in larger vessels, is associated with flame-like amyloid-beta ($A\beta$) deposits that may extend beyond the vessel wall and radiate into the neuropil, a phenomenon also known as "dyschoric angiopathy." $A\beta$ deposits in AD, parenchymal as well as (cap)CAA and dyschoric angiopathy, are associated with a local inflammatory reaction, including activation of microglial cells and astrocytes that, among others, produce cytokines and reactive oxygen species. This neuroinflammatory reaction may account for at least part of the cognitive decline. In previous studies we observed that small heat shock proteins (sHsps) are associated with $A\beta$ deposits in AD. In this study the molecular chaperones Hsp20, HspB8 and HspB2B3 were found to colocalize with CAA and capCAA in AD brains. In addition, Hsp20, HspB8 and HspB2B3 colocalized with intercellular adhesion molecule 1 (ICAM-1) in capCAA-associated dyschoric angiopathy. Furthermore, we demonstrated that Hsp20, HspB8 and HspB2B3 induced production of interleukin 8, soluble ICAM-1 and monocyte chemoattractant protein 1 by human leptomenigeal smooth muscle cells and human brain astrocytes *in vitro* and that Hsp27 inhibited production of transforming growth factor beta 1 and CD40 ligand. Our results suggest a central role for sHsps in the neuroinflammatory reaction in AD and CAA and thus in contributing to cognitive decline.

Introduction

In Alzheimer's disease (AD) amyloid- β ($A\beta$) is deposited as plaques in the parenchyma, but also as cerebral amyloid angiopathy (CAA) (Selkoe, 1991). Capillary $A\beta$ deposits (capCAA) correlates with severity of AD pathology and clinical deterioration, whereas larger vessel CAA does not (Attems and Jellinger, 2004; Eurelings et al., 2010). Furthermore, in capCAA, flame-like $A\beta$ deposits may extend beyond the vessel wall and radiate into the neuropil, a phenomenon known as "dyschoric angiopathy" (Attems et al., 2011). Neuropathological examination of (cap)CAA reveals activated microglia and astrocytes surrounding these lesions (McGeer and McGeer, 1995; Yamada et al., 1996; Akiyama et al., 2000; Rozemuller et al., 2005; Richard et al., 2010; Attems et

al., 2011) and colocalization of inflammatory factors such as intercellular adhesion molecule 1 (ICAM-1) (Verbeek et al., 1996).

In AD brains, an inflammatory reaction may occur in conjunction with $A\beta$ deposits, characterized by the increased expression of, among others, transforming growth factor β 1 ($TGF-\beta_1$) (Wyss-Coray et al., 1997), interleukin (IL) 1 β , IL-8, monocyte chemoattractant protein 1 (MCP-1), ICAM-1 and CD40 ligand (Akiyama et al., 2000; Calingasan et al., 2002; Sokolova et al., 2009; Heneka et al., 2010). This inflammatory reaction may occur in response to the presence of extracellular $A\beta$ or phagocytosis of $A\beta$ deposits by microglia (Malm et al., 2010). Inflammation, both directly via interaction of activated glial cells and indirectly via secreted neurotoxic mediators may compromise neuronal function. Indeed, transgenic mice with extensive CAA showed cognitive decline as a result of increased microglial activation and neuroinflammation (Xu et al., 2007), which could be reduced by treatment with the anti-inflammatory drug minocycline, and thereby improved cognition (Fan et al., 2007). Epidemiological studies demonstrated that most patients with CAA-related inflammation show at least a partial clinical improvement to high dose corticosteroids or other immunosuppressive agents (Chung et al., 2011). Thus both human and mouse studies provide evidence for a contribution of $A\beta$ to CAA-related neuroinflammation and subsequent cognitive decline. However, the neuroinflammatory reaction in AD patients may be more complex.

In AD, several macromolecules colocalize with plaques and CAA, including small heat shock proteins (sHsps) (Wilhelmus et

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al., 2006b,c). Previous research showed that expression of α B-crystallin and Hsp27 is increased in reactive astrocytes of AD brains (Renkawek et al., 1993, 1994; Wilhelmus et al., 2006c). Hsp20 codeposited with A β in diffuse and classic plaques and HspB2 with classic plaques and CAA (Wilhelmus et al., 2006c). Moreover, HspB8 colocalized with classic plaques in AD and with CAA in hereditary cerebral hemorrhage with amyloidosis of the Dutch type (HCHWA-D) (Wilhelmus et al., 2006b). Furthermore, we observed that these sHsps, but not A β , induced IL-6 production in various cerebral cell cultures (Wilhelmus et al., 2009), suggesting that sHsps may play a pivotal role in inducing neuroinflammation. Therefore, we investigated the association of sHsps with (cap)CAA and CAA-associated inflammation in postmortem brain and investigated the effect of selected sHsps on the production of inflammatory factors by cultured human leptomeningeal smooth muscle cells and astrocytes *in vitro*.

Materials and Methods

Reagents. Lyophilized A β _{1–42} (95% pure) and A β _{1–40} (98% pure) were purchased from Quality Controlled Biochemicals. A β with the “Dutch” mutation (22 Glu→Gln, D-A β _{1–40}, 96% pure) was purchased from 21st Century Biochemicals. D-A β _{1–40} was used as a model peptide, because it causes CAA in hereditary cerebral hemorrhage with amyloidosis of the Dutch type (HCHWA-D) (Davis and Van Nostrand, 1996; Verbeek et al., 1997; Wilhelmus et al., 2007). A β _{1–42}, A β _{1–40}, and D-A β _{1–40} were dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (Sigma-Aldrich), air-dried, and dissolved in DMSO at a concentration of 5 mM. Aliquots of the solutions were stored at –80°C.

Autopsy material. Patient selection was based on neuropathological findings at autopsy. Tissue samples were selected for the presence of capCAA (Richard et al., 2010). For immunohistochemistry, tissue samples from the occipital neocortex from 3 AD patients with capCAA (age 76 ± 12 years; postmortem delay 5:53 ± 2:09 h) were obtained after rapid autopsy and immediately frozen in liquid nitrogen. Informed consent was obtained according to European guidelines. Table 1 provides an overview of the diagnosis, Braak stage, CERAD score, CAA grade, age, postmortem interval (PMI) and gender. Neuropathological evaluation was performed on frozen tissue from occipital pole cortex. CAA score was defined as follows: severe (+++), moderate (++) , mild (+) as previously described (Richard et al., 2010). Staging of AD was evaluated according to the criteria established by Braak and Braak and CERAD (Braak and Braak, 1991; Mirra et al., 1991).

Immunohistochemistry. In Table 2 the primary antibodies used in this study are listed. Unless mentioned otherwise, we used biotin-labeled horse anti-mouse antibodies (Vector Laboratories) as the detection antibody for mouse monoclonal antibodies, or biotin-labeled goat-anti-rabbit antibodies (Vector Laboratories) in the case of rabbit polyclonal antibodies.

Serial cryosections (4 μ m) were air-dried and fixed in acetone for 5 min. Endogenous peroxidase activity was blocked using acetone containing 0.15% H₂O₂ for 5 min. Sections were preincubated for 30 min with 20% animal serum, the type of which was determined by the specific biotin-labeled antibody used. Subsequently, sections were incubated overnight at 4°C with primary antibody (Table 2), with secondary antibodies for 60 min at room temperature, and with the avidin-biotin complex according to the manufacturer's description. Between incubation steps, sections were extensively washed using PBS. 3-Amino-9-ethyl carbazole (AEC) was used as a chromogen. After a short rinse in tap water the preparations were incubated with hematoxylin for 1 min and thoroughly washed with tap water for 10 min. Finally, sections were mounted with Imsol mount (Klinipath). All antibodies were diluted in PBS containing 1% bovine serum albumin (BSA), which also served as a negative control. At regular intervals, sections were stained with anti-A β to allow for topographical alignment of CAA with the stains for sHsps and inflammatory factors.

For double immunostaining of A β and/or astrocytes/microglia, sections were stained using the EnVision method as previously described

Table 1. Overview of the origin of astrocyte and HBP cell cultures and tissue sections

Number	Diagnosis	Age (years)	PMI (h:m)	Gender	NFT stage (Braak)	Plaque score (CERAD)	CAA grade	Cell type
Cell culture								
1	AD	84	6:30	F	V	C	N.D.	Astrocyte
2	AD	92	7:00	F	V	0	N.D.	Astrocyte
3	Control	76	4:40	F	I	B	N.D.	Astrocyte
4	AD	77	3:00	M	VI	C	++	SMC
5	Parkinson	91	4:00	F	V	C	+++	SMC
6	Control	83	4:00	F	III	B	–	SMC
IHC								
7	AD + CAA	65	7:20	M	V	C	+++	N.A.
8	AD + CAA	89	6:55	F	V	C	+++	N.A.
9	AD + CAA	74	3:25	M	VI	C	+++	N.A.
10	Control	81	3:45	M	I	0	–	N.A.
11	Control	71	6:05	F	III	C	–	N.A.
12	Control	92	6:25	F	III	A	+	N.A.

Overview of the cases used in this study. IHC, Tissue used for immunohistochemistry; F, female; M, male; N.D., not determined; N.A., not applicable. Grading of AD (Braak scores) was performed as described in Materials and Methods.

Table 2. Primary antibodies used in this study

Primary antibody	Antigen	Species raised in	Dilution	Source (reference)
6C6	A β	Mouse	1:250	Dr. Schenk, Elan Pharmaceuticals
GFAP	GFAP	Mouse	1:10	Monosan, Sanbio
LN3	HLA-DR	Mouse	1:50	eBioscience
MAB208	IL-8	Mouse	1:100	R&D Systems
LS-C8642	MCP-1	Mouse	1:100	Lifespan Biosciences
CL203	ICAM-1	Mouse	1:500	(Verbeek et al., 1994a)
PN-E12	ICAM-1	Mouse	1:25	(Verbeek et al., 1994a)
Pab-aBcr	α B-Crystallin	Rabbit	1:500	(den Engelsman et al., 2003)
1426	Hsp27	Rabbit	1:2000	Abcam
VDK-p20	Hsp20	Rabbit	1:4000	(van de Klundert et al., 1998)
J92	HspB8	Rabbit	1:2000	(Verschuure et al., 2003)
F34	HspB2	Rabbit	1:2000	(Verschuure et al., 2003)

(Richard et al., 2010). Briefly, sections were incubated overnight at 4°C with primary antibodies (Table 2), followed by incubation with EnVision HRP. EnVision 3,3-diaminobenzidine (DAB) was used as chromogen. Antibodies were diluted in PBS/1% BSA, which also served as a negative control. After each incubation, slides were extensively washed with PBS. For the detection of fibrillar A β in plaques and CAA, sections were stained with Congo Red (0.5% in Ethanol 80%, 3% NaCl) for 20 min at room temperature. Finally, sections were mounted with DePeX mounting medium Gurr.

Expression and purification of recombinant proteins. Recombinant human α B-crystallin and HspB2B3 were expressed and purified as described previously (Wilhelmus et al., 2006a). Since it was demonstrated that HspB2 and HspB3 form functional heterogeneous complexes of 150 kDa (Sugiyama et al., 2000), both proteins were expressed together in one vector. Coding sequences for the following sHsps were ligated into the pET-46 Ek/LIC vector which also introduces a N-terminal His-tag sequence (Novagen, Merck Chemicals Ltd): human Hsp27, human Hsp20, and human HspB8. Hsp20 and HspB8 were expressed in Rosetta(DE3)pLysS competent cells (Novagen). Hsp27 was expressed in its BL21(DE3)pLysS competent cells (Novagen). A culture inoculated from a single colony was induced by addition of 0.25 mM isopropyl-thio- β -D-galactopyranoside (IPTG) for 4 h at 37°C, with the exception of Hsp20, which was induced for 16 h at 25°C. After incubation with IPTG, cells were harvested and lysed by sonication in native lysis buffer (NLB; 50 mM NaH₂PO₄, 300 mM NaCl, pH 8.0) supplemented with EDTA-free protease inhibitor (Roche Applied Science). Insoluble proteins were removed by centrifugation. All sHsps were purified over a Ni-NTA agarose

column (Qiagen) using NLB supplemented with increasing concentrations of imidazole (Sigma-Aldrich Chemie BV) ranging from 20 up to 250 mM. After desalting, sHsps were concentrated in 10 mM Tris, 50 mM NaCl, pH 7.4 buffer by using an iCON concentrator spin column (Thermo Fisher Scientific Inc.). Protein concentration was determined using a BCA Protein Assay kit according to the manufacturer's description (Thermo Fisher Scientific). Purified sHsps were aliquoted and stored at -80°C . Purity of the preparations was $\geq 95\%$.

Cell culture. Smooth muscle cells (SMCs) and astrocytes were both isolated from human brain tissue obtained after rapid autopsy, as described previously (De Groot et al., 1997; Verbeek et al., 1994b; Veerhuis et al., 1998) (Table 1). Diagnosis and grading of patients was performed according to the criteria mentioned previously. SMCs were maintained in Eagle's modification of essential medium (EMEM; PAA Laboratories GmbH), supplemented with 10% human serum (Lonza BioWhittaker Benelux BV), 20% fetal bovine serum (PAA Laboratories GmbH), recombinant basic fibroblast growth factor (1 ng/ml) and gentamycin (2.5 $\mu\text{g}/\text{ml}$). Astrocytes were maintained in a mixture (1:1 v/v) of DMEM and Ham's F10 supplemented with 10% fetal bovine serum, 2 mM L-glutamine (Cambrex) and gentamycin (2.5 $\mu\text{g}/\text{ml}$). All primary cell cultures were cultured at 37°C , 5% CO_2 and 90% relative humidity. Although the cultured astrocytes and SMCs were derived from different clinical backgrounds (i.e., either AD or control patients), we did not observe differences with respect to their responses in the various types of experiments.

Cell passages 3–9 were used for the experiments. Control cells incubated with EMEM or DMEM/Ham's F10 and 0.1% BSA (serum-free medium) demonstrated normal morphology. Experiments were performed at least twice.

ELISA. Duplicate wells (12-well plate; Corning Inc) with cultured cells were preincubated with serum-free medium for a minimum of 4 h. Subsequently, cells were incubated with 12.5 μM synthetic $\text{A}\beta_{1-40}$, $\text{A}\beta_{1-42}$, D- $\text{A}\beta_{1-40}$ or 12.5 μM purified sHsps (αB -crystallin, Hsp27, Hsp20, HspB8 and HspB2B3) at 37°C and 5% CO_2 . This concentration was the lowest concentration to induce a significant effect across all sHsps tested. In addition, the same concentration of $\text{A}\beta$ induced biological effects in cultured SMCs and astrocytes (Verbeek et al., 1997; Bruinsma et al., 2010) and was therefore chosen in the experiments. Lipopolysaccharide (LPS; 055:B5; Sigma-Aldrich) stimulation (1 $\mu\text{g}/\text{ml}$) was used as a positive control for inducing cytokine production. After 48 h of incubation, supernatants and cell lysates (solubilized in RIPA; 50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS in Milli-Q) were collected, centrifuged at 13,200 rpm for 10 min at 4°C and stored at -80°C until further use. To exclude endotoxin contamination, potentially present in the purified sHsp samples, coincubation with polymixin B (PMB; Sigma-Aldrich; 10 $\mu\text{g}/\text{ml}$) was used to inhibit LPS-mediated inflammation. In these experiments, PMB was preincubated with both the LPS and the sHsps for 1 h at 37°C before addition to the cells.

IL-8, ICAM-1, MCP-1, CD40 ligand, and TGF- β_1 levels in cell culture supernatant were measured using commercial enzyme immunoassay kits according to the manufacturer's instructions (DuoSet ELISA, R&D Systems). To correct for differences in cell density, inflammatory factor production was normalized to protein content of the cell lysate which was determined by using a BCA Protein Assay kit according to the manufacturer's description (Thermo Fisher Scientific Inc.). Concentrations of the various inflammatory factors were expressed as pg/mg protein.

Statistical analysis. Statistical analyses were performed using an independent sample *t* test in SPSS 14.0 for Windows software. Statistical significance was set at $p < 0.05$.

Results

Inflammatory cells in AD brains with severe CAA pathology, capCAA, and dyschoric angiopathy

Double staining for GFAP and Congo red demonstrated the presence of astrocytes around $\text{A}\beta$ -laden vessels, with a more prominent GFAP staining around the larger vessels (Fig. 1A,C). Clusters of HLA-DR-positive microglia were also observed around larger ves-

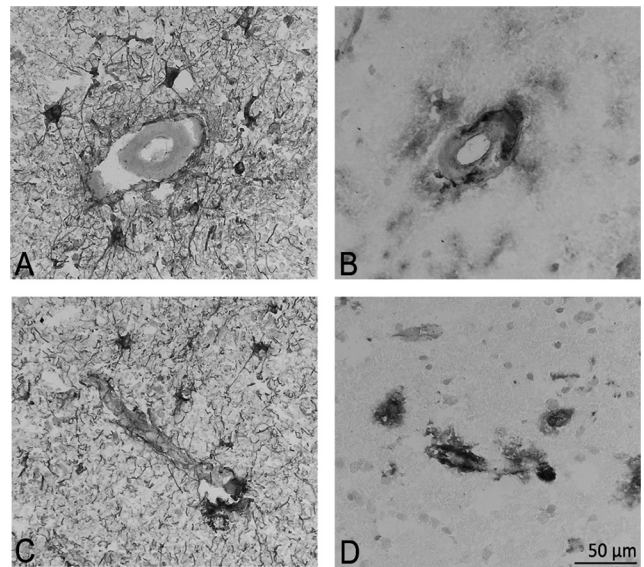


Figure 1. Immunohistochemical staining of GFAP (A, C) and microglial cell marker (LN3; B, D) in CAA (A, B) and capCAA (C, D). All sections are double stained with Congo Red. GFAP-positive reactive astrocytes are present around both larger vessels and capillaries (A, C). Microglial activation is associated with both large vessels and capillaries, however more prominent around the latter (B, D).

sels and capillaries laden with $\text{A}\beta$ and were particularly prominent around the capillaries (Fig. 1B,D).

Expression of sHsps in AD brains with severe CAA pathology, capCAA, and dyschoric angiopathy

In control brains, αB -crystallin staining was observed in glial cells (Fig. 2B). Furthermore, leptomeningeal vessels as well as a few astrocytes in the white matter were also stained (data not shown). Hsp27 staining in control brains was limited to leptomeningeal vessels (data not shown), but not observed in parenchymal vessels (Fig. 2D). Hsp20 staining in control brains was occasionally observed in astrocytes in white and gray matter (data not shown). Weak immunoreactivity of Hsp20 was also observed in large parenchymal (Fig. 2F, open arrow) and leptomeningeal vessels (data not shown). HspB8 staining in control brains was observed in astrocytes in both gray and white matter (Fig. 2H, arrow) and in cerebrovascular cells of large parenchymal vessels (Fig. 2H, open arrow). Furthermore, weak staining for HspB2 was observed in cerebrovascular cells in large parenchymal vessels (Fig. 2J, open arrow) and in an occasional astrocyte or microglial cell in the brain parenchyma (data not shown). IL-8 (Fig. 2L) and MCP-1 (Fig. 2P) staining was not observed in normal parenchymal or leptomeningeal vessels in control brains, whereas ICAM-1 staining was observed in endothelial cells in control brains (Fig. 2N, arrow).

In AD brains with severe (cap)CAA pathology, the anti- $\text{A}\beta$ antibody (6C6) stained $\text{A}\beta$ -affected vessels and capillaries (Fig. 3A,B). In addition, dyschoric angiopathy (Fig. 3A) was often observed. The antibodies directed against αB -crystallin, Hsp27, Hsp20, HspB8 and HspB2 showed different staining patterns for these sHsps in CAA, capCAA and dyschoric angiopathy. Representative staining results are shown in Figure 3 and a summary of the immunohistochemical results is shown in Table 3. Anti- αB -crystallin antibody stained reactive astrocytes and activated microglia associated with CAA (Fig. 3C) and capCAA (Fig. 3D). Hsp27 staining was not observed in gray matter (Fig. 3E,F), but was occasionally observed in reactive astrocytes and microglia in

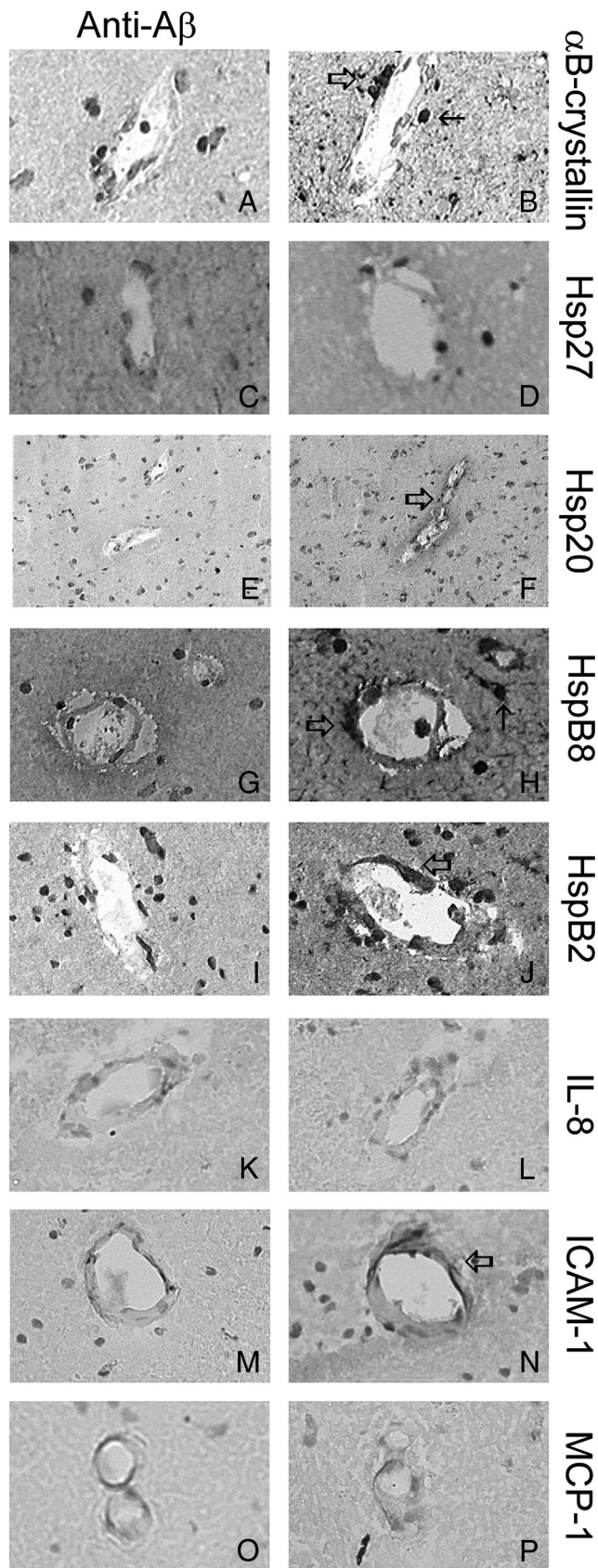


Figure 2. Immunohistochemical staining of A β (**A, C, E, G, I, K, M, O**), sHsps (**B, D, F, H, J**; open arrow), and inflammatory factors (**L, N, P**; open arrow) in or near normal vessels in the occipital cortex of control patients. Serial sections: **A, B; C, D; E, F; G, H; I, J; K, L; M, N; O, P**. Anti- α B-crystallin antibodies stained glial cells throughout the cortex (**B**, arrow). Anti-Hsp27

the white matter (data not shown). In addition, reactive astrocytes surrounding CAA were immunopositive for Hsp20 (Fig. 3G) and occasionally Hsp-20-positive reactive astrocytes were observed surrounding capCAA (data not shown). Anti-HspB8 stained reactive astrocytes and microglia in both white and gray matter and occasional immunoreactivity of HspB2 was observed with reactive astrocytes and microglia. Whereas HspB2 (Fig. 3L) colocalized with A β in capCAA and CAA, no immunostaining for α B-crystallin (Fig. 3C,D), Hsp27 (Fig. 3E, F), Hsp20 (Fig. 3G,H) and HspB8 (Fig. 3I,J) was observed. Furthermore, colocalization of Hsp20 (Fig. 3G), HspB8 (Fig. 3I) and HspB2 (Fig. 3K), but not of α B-crystallin (Fig. 3C) and Hsp27 (Fig. 3E), with A β surrounding CAA and capCAA (dyshoric angiopathy) was observed.

Expression of inflammatory factors in AD brains with severe CAA pathology, capCAA, and dyshoric angiopathy

ICAM-1 was associated with dyshoric A β deposits surrounding CAA and capCAA, but not with the A β deposits within the vessel wall (Fig. 3O,P), suggesting colocalization of Hsp20, HspB8 and HspB2 with ICAM-1 in dyshoric angiopathy near (cap)CAA. Although ICAM-1 has also been detected in a soluble form (Harning et al., 1991; Rothlein et al., 1991), it is mainly described as a membrane-bound cell adhesion molecule. Therefore, the ICAM-1 staining we observed is very likely due to membrane-bound ICAM-1. In earlier studies an increase in IL-8 and MCP-1 in AD brains was observed after soluble protein extraction of human brain lysates or in paraffin-embedded formalin-fixed tissue (Akiyama et al., 2000; Calingasan et al., 2002; Sokolova et al., 2009). In the present study, no IL-8 and MCP-1 was detectable in tissue from AD patients with severe CAA pathology (Fig. 3M,N,Q,R). In cryosections, these soluble factors might escape detection due to mild fixation of the tissue.

sHsps induce the production of inflammatory factors by human leptomenigeal smooth muscle cells and human brain astrocytes

Our immunohistochemical studies suggest that both A β and sHsps accumulate in or near (cap)CAA, where an inflammatory reaction is observed as well. Since human leptomenigeal SMCs are already early affected by A β in CAA (Vinters et al., 1996), we studied whether A β or sHsps were able to induce production of cytokines and other inflammatory factors by cultured SMCs. In addition, since reactive astrocytes colocalize near (cap)CAA (Fig. 1), we also studied the potential of A β and sHsps to induce an inflammatory reaction in human brain astrocytes.

Neither A β _{1–40}, A β _{1–42}, nor D-A β _{1–40} (12.5 μ M) induced production of IL-8, ICAM-1, MCP-1, TGF- β ₁ or CD40 ligand by SMCs or astrocytes, above control levels (Figs. 4, 5). Incubation of 1 μ g/ml LPS induced IL-8 production (Fig. 4A; $p < 0.05$) by SMCs, but not production of soluble ICAM-1 (sICAM-1; Fig.

←

antibodies were only occasionally immunoreactive with leptomenigeal vessels (data not shown), but not with parenchymal vessels (D). Hsp20 was occasionally demonstrated in astrocytes in normal brains (data not shown). Weak immunoreactivity of Hsp20 was also observed in large parenchymal (F, open arrow) and leptomenigeal vessels. HspB8 staining was observed in astrocytes in both gray and white matter (H, arrow) and in cerebrovascular cells of large parenchymal vessels (J, open arrow). Weak staining for HspB2 was observed in cerebrovascular cells in large parenchymal vessels (J, open arrow) and in an occasional astrocyte or microglial cell in the brain parenchyma (data not shown). Furthermore, anti-IL-8 (L) and anti-MCP-1 (P) staining was absent from normal parenchymal or leptomenigeal vessels. The anti-ICAM antibodies stained endothelial cells (N, arrow). Original magnification 200 \times .

4B), MCP-1 (Fig. 4C) and TGF- β_1 (Fig. 4D). Furthermore, incubation of 1 $\mu\text{g}/\text{ml}$ LPS induced IL-8 (Fig. 5A; $p < 0.01$), sICAM-1 (Fig. 5B; $p < 0.01$) and MCP-1 (Fig. 5C; $p < 0.05$) production by astrocytes, but not production of TGF- β_1 (Fig. 5D) and CD40 ligand (Fig. 5E). After preincubation and coinubation of LPS with PMB, production of inflammatory factors by SMCs and astrocytes returned to control levels (Fig. 5; $p < 0.05$), indicating that PMB efficiently inhibited the LPS-induced inflammatory reaction.

Incubation of SMCs with 12.5 μM Hsp20, HspB8 or HspB2B3 resulted in a strong induction of IL-8 (Fig. 4A; $p < 0.01$), sICAM-1 (Fig. 4B; Hsp20, HspB2B3, $p < 0.001$; HspB8, $p < 0.01$) and MCP-1 (Fig. 4C; Hsp20, HspB2B3, $p < 0.001$; HspB8, $p < 0.01$) secretion, but not of TGF- β_1 (Fig. 4D). In contrast to the increased levels of IL-8 production after incubation with 12.5 μM Hsp27 (Fig. 4A; $p < 0.001$), decreased ($p < 0.01$) TGF- β_1 production was observed after Hsp27 treatment compared with control (Fig. 4D). In addition, incubation with Hsp27 did not induce levels of sICAM-1 (Fig. 4B) and MCP-1 (Fig. 4C) above control levels in SMCs. Furthermore, SMCs did not seem to secrete CD40 ligand, since after incubation of these cells with either A β_{1-40} , A β_{1-42} , and D-A β_{1-40} or sHsps, the concentrations of CD40 ligand remained below the detection level (data not shown).

Incubation of astrocytes with 12.5 μM Hsp20, HspB8 or HspB2B3 also resulted in a strong induction of IL-8 (Fig. 5A; $p < 0.001$), sICAM-1 (Fig. 5B; $p < 0.001$) and MCP-1 (Fig. 5C; $p < 0.001$) secretion, but not of TGF- β_1 (Fig. 5D). In addition, only 12.5 μM HspB8 and HspB2B3 induced CD40 ligand production by astrocytes (Fig. 5E; $p < 0.05$ and $p < 0.01$ respectively). Comparable to the effects of Hsp27 in SMCs, IL-8 production by astrocytes was also increased after incubation with 12.5 μM Hsp27 (Fig. 5A; $p < 0.01$), however, Hsp27 induced a decrease in the production of TGF- β_1 (Fig. 5D; $p < 0.001$) and CD40 ligand (Fig. 5E; $p < 0.01$) compared with control. Furthermore, incubation with Hsp27 did not result in levels of sICAM-1 (Fig. 5B) and MCP-1 (Fig. 5C) above control levels in astrocytes.

To exclude LPS contamination, potentially present in the purified sHsp samples, we coinubated cell samples with PMB and sHsps to inhibit potential LPS-mediated inflammation. After preincubation and coinubation of LPS with PMB, production of inflammatory factors by SMCs and astrocytes returned to control levels (Fig. 6A,B; $p < 0.05$), indicating that PMB efficiently inhibited the LPS-induced inflammatory reaction. Whereas 12.5 μM αB -crystallin seemed to induce IL-8 secretion by SMCs and astrocytes, this effect was completely abolished by preincubation and coinubation with PMB (Fig. 6A,B; $p < 0.05$), indicating that traces of LPS in the αB -crystallin preparations are responsible for the observed effects. In contrast, PMB had no, or only a minimal effect,

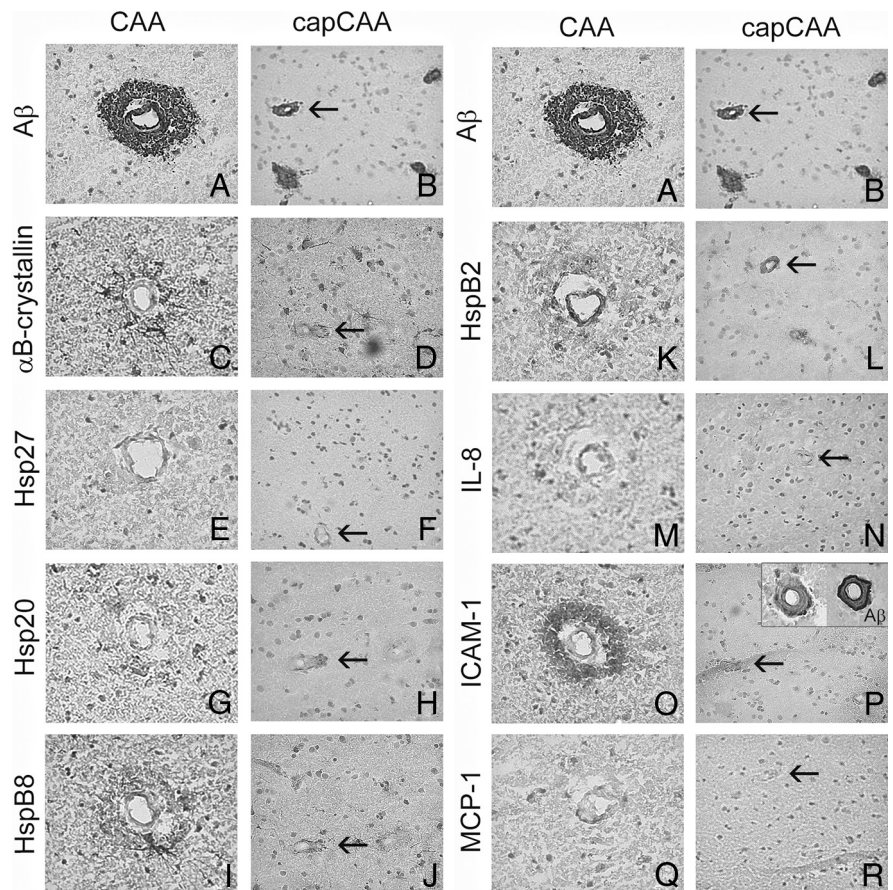


Figure 3. Immunohistochemical staining of sHsps and inflammatory factors in or near CAA vessels (A, C, E, G, I, K, M, O, Q; serial sections) and capCAA (B, D, F, H, J, L, N, P, R; serial sections; arrows indicate the same capillary in each panel to serve as a reference to allow optimal comparison with other panels) in the occipital cortex of AD brains. The anti-A β antibody 6C6 stained CAA, capCAA, and dyschoric angiopathy in AD brains (A, B; to allow optimal comparison with other stains, these panels are shown twice). Both anti- αB -crystallin (C, D) and anti-Hsp27 (E, F) antibody staining was absent in A β deposits. No anti-Hsp20 and anti-HspB8 staining was observed in CAA and capCAA; however, colocalization of Hsp20 and HspB8 (G, H; I, J) with dyschoric angiopathy (A β surrounding CAA) was observed. The presence of HspB2 in CAA (K) and capCAA (L) of AD brains with severe CAA was observed, but also colocalization of HspB2 with dyschoric angiopathy (K) was observed. The anti- αB -crystallin (C), anti-Hsp20 (G), and anti-HspB8 antibodies (I) stained reactive astrocytes and microglia associated with CAA. Occasionally, immunoreactivity of HspB2 with reactive astrocytes and microglia was observed. Furthermore, anti-IL-8 (M, N, arrow) and anti-MCP-1 (Q, R, arrow) staining was absent from CAA and capCAA vessels. In addition, ICAM-1 was associated with dyschoric angiopathy, but not with the A β deposits in the vessel wall (O, P, arrow; inset, ICAM-1 staining (left) and A β staining (right) of another capCAA vessel). Original magnification 200 \times .

Table 3. Summary of the immunohistochemical results

	capCAA	CAA	Dyschoric angiopathy
αB -crystallin	0%	0%	0%
Hsp27	0%	0%	0%
Hsp20	10%	0%	90%
HspB8	10%	0%	100%
HspB2	80%	100%	95%

Quantification of the percentages of A β deposits that are stained by the sHsp antibodies in all patients (average of $n = 3$ patients).

on the production levels of IL-8 by SMCs and astrocytes induced by the other sHsps samples (Fig. 6A,B), suggesting a strong inducing effect of these sHsps on cytokine production.

Discussion

The main findings of this study are as follows: 1) Hsp20, HspB8 and HspB2 are associated with (cap)CAA and dyschoric angiopathy in AD brains with severe CAA pathology; 2) these sHsps colocalize

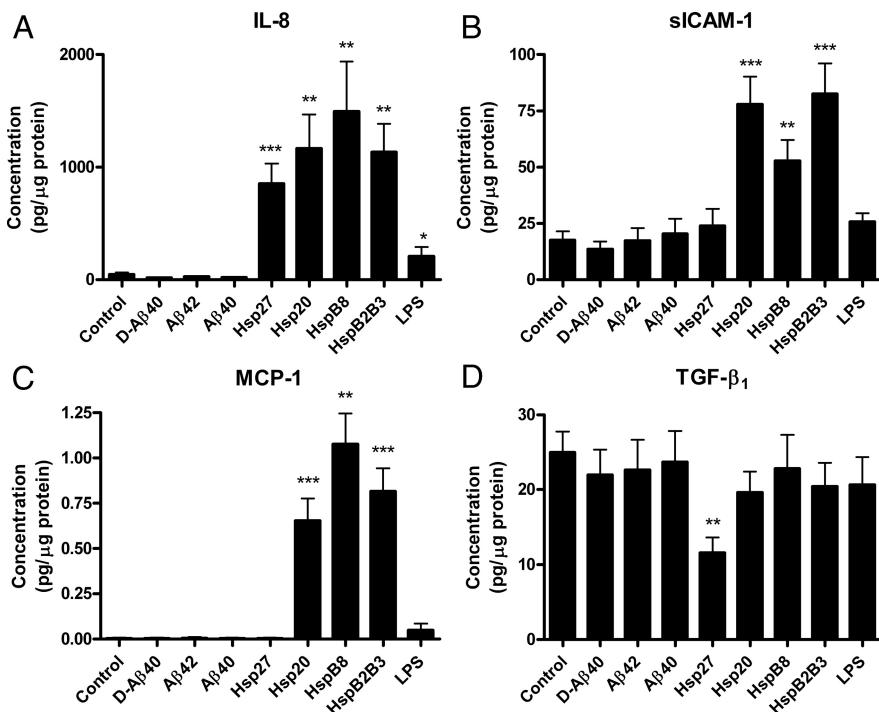


Figure 4. Production of inflammatory factors by human brain leptomeningeal SMCs. Cultured SMCs were incubated with 12.5 μM D-A β_{40} , A β_{42} , A β_{40} , Hsp27, Hsp20, HspB8, HspB2B3, or 1 $\mu\text{g}/\text{ml}$ LPS for 2 d at 37°C. Supernatant was collected and IL-8 (A), sICAM-1 (B), MCP-1 (C), and TGF- β_1 (D) concentrations were measured using ELISA. Statistical analysis was performed using an independent sample *t* test. The level of significance of the difference compared with control is indicated as follows: **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *p* > 0.05 is not indicated. Means \pm SEM are shown.

with ICAM-1 in dyschoric angiopathy near (cap)CAA; 3) sHsps (especially Hsp20, HspB8 and HspB2B3), but not A β , increased secretion of IL-8, sICAM-1 and MCP-1 by cultured human leptomeningeal SMCs and human brain astrocytes; 4) Hsp27 reduced secretion of TGF- β_1 by SMCs and astrocytes and CD40 ligand secretion by astrocytes.

The observed astrocytic and microglial activation, indicative of an inflammatory reaction, around (cap)CAA in this report is in line with earlier studies (Richard et al., 2010; Attems et al., 2011). In addition, colocalization of Hsp20, HspB8 and HspB2, but not αB -crystallin and Hsp27, with (cap)CAA and dyschoric angiopathy near (cap)CAA is also in line with our previous studies in which colocalization of these sHsps was found with CAA and/or parenchymal deposits of A β (Wilhelmus et al., 2006b,c, 2009). It has been suggested that Hsp20, HspB8 and HspB2 are predominantly associated with fibrillar A β *in vivo* (Wilhelmus et al., 2006b,c, 2009) and our results suggest a similar association of these sHsps with fibrillar A β in (cap)CAA and with dyschoric angiopathy near (cap)CAA. Thus, Hsp20, HspB8 and HspB2 are clearly associated with various types of A β deposits in AD brains and may participate in the local inflammatory reaction.

CAA-related inflammation is of clinical importance since patients with this type of pathology present with cognitive decline, seizures and headaches, that improve upon anti-inflammatory treatment (Eng et al., 2004; Kinnecom et al., 2007; Chung et al., 2011). In addition, it has been suggested that especially capCAA with spreading of the A β deposits into the neuropil could contribute to a rapid clinical deterioration (Eurelings et al., 2010), suggesting an important role of capCAA and dyschoric angiopathy, rather than plaques and CAA, in cognitive decline. In AD, A β has been shown to trigger neuroinflammation by e.g., activation of the complement system (Heneka et al., 2010), and activation of

microglia and astrocytes (Tan et al., 1999; Heneka et al., 2010). In addition, the pro-inflammatory factors IL-8, MCP-1 and CD40 ligand are increased in AD (Akiyama et al., 2000; Calingasan et al., 2002; Sokolova et al., 2009) and ICAM-1 and TGF- β_1 colocalize with A β deposits (Verbeek et al., 1996; Wyss-Coray et al., 1997), suggesting that A β may induce the expression of these inflammatory factors. In contrast, however, the results of the present study suggest that sHsps may be much more potent inducers of an inflammatory reaction in AD brains than A β . Interestingly, the proinflammatory effect of these sHsps does not seem to be limited to a single parameter such as IL-6 (Wilhelmus et al., 2009), but includes multiple inflammatory factors that are observed *in vivo*. We obtained *in vivo* evidence for the colocalization of sHsps near sites of A β -associated inflammation in the cerebral vasculature and demonstrated *in vitro* that Hsp20, HspB8 and HspB2B3 induced production and secretion of IL-8, MCP-1 and sICAM-1 in SMCs and astrocytes, with little or no effect of Hsp27 and no effect of αB -crystallin on these factors. These results are in line with, and extend, our previous findings that sHsps may be among the key mediators of the inflam-

matory reactions associated with (cap)CAA, since they also induced IL-6 production in cultured SMCs, astrocytes and microglia (Wilhelmus et al., 2009). Interestingly, our results suggest that those sHsps that occur extracellularly in plaques or dyschoric angiopathy and (cap)CAA (i.e., Hsp20, HspB8 and HspB2B3) have a potent proinflammatory effect, indicating that especially these sHsps are important in the inflammatory reaction in CAA. Interestingly, HspB8 and HspB2B3 induced CD40 ligand expression in astrocytes, but not in SMCs, suggesting that the reported upregulation of CD40 ligand in AD (van der Wal et al., 1993; Calingasan et al., 2002) might be due to astrocytic production. In contrast to these findings, however, Hsp20, HspB8 and HspB2B3 did not induce production of TGF- β_1 in both SMCs and astrocytes, although these factors are also reported to be upregulated in AD (van der Wal et al., 1993; Calingasan et al., 2002). This suggests that this cytokine might be upregulated by alternative mechanisms or cell types.

Hsp27 is a member of the sHsp family with remarkable capacities compared with the other sHsps tested in this study. Hsp27 was able to induce IL-8 and inhibit TGF- β_1 production in both human SMCs and astrocytes *in vitro*. Furthermore, Hsp27 inhibited CD40 ligand production in astrocytes. Interestingly, IL-8 is a proinflammatory cytokine and TGF- β_1 can act as an anti-inflammatory factor by inhibition of local inflammation resulting in reduced tissue injury that may occur as a response to inflammation (Wahl, 1992). Furthermore, the interaction of CD40 ligand with its receptor has been implicated in the modulation of anti-inflammatory responses (Liu et al., 2010). Thus, Hsp27 may exert a proinflammatory effect, both through inducing a proinflammatory reaction (e.g., IL-8) and reducing the production of the anti-inflammatory factors (e.g., CD40 ligand and TGF- β_1) (Liu et al., 2010). In addition, Hsp27 may also function

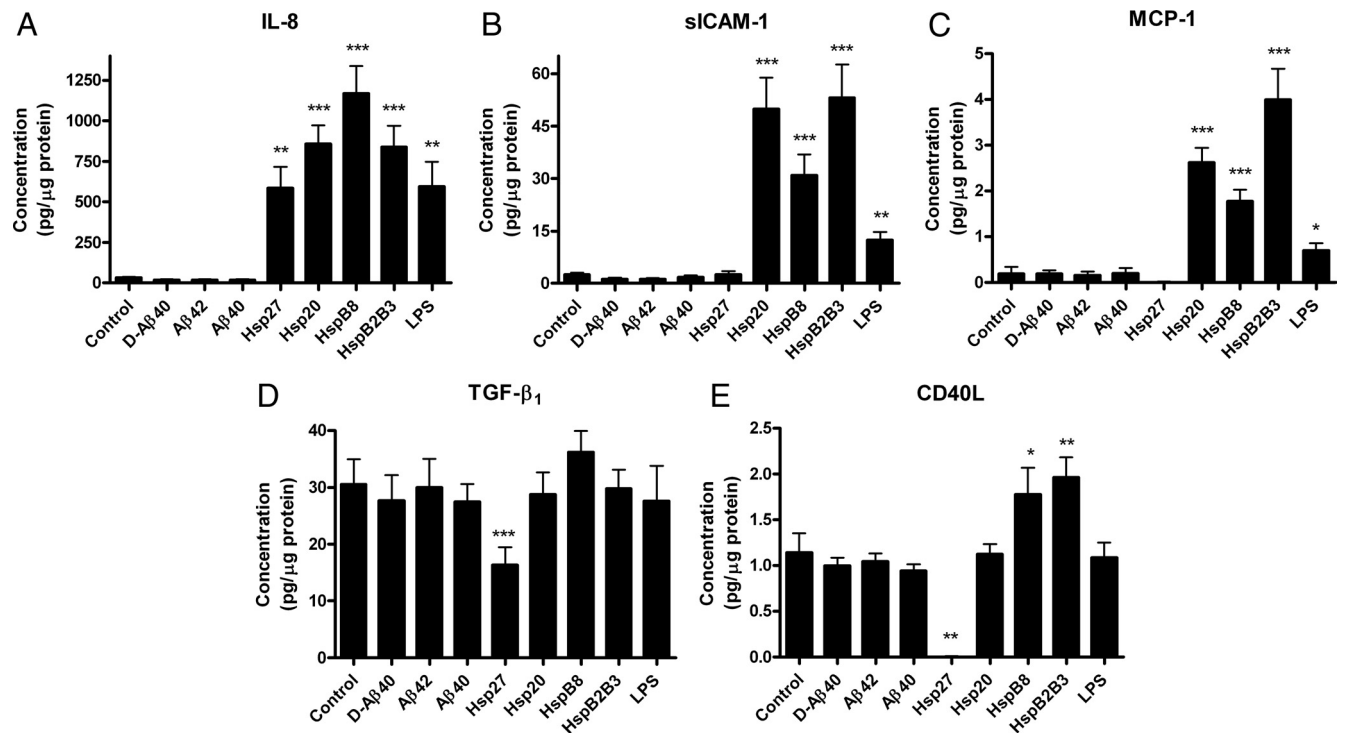


Figure 5. Production of inflammatory factors by human brain astrocytes. Cultured astrocytes were incubated with 12.5 μM D-Aβ₄₀, Aβ₄₂, Aβ₄₀, Hsp27, Hsp20, HspB8, HspB2B3, or 1 μg/ml LPS for 2 d at 37°C. Supernatant was collected and IL-8 (A), sICAM-1 (B), MCP-1 (C), TGF-β₁ (D), and CD40 ligand (E) concentrations were measured using ELISA. Statistical analysis was performed using an independent sample *t* test. The level of significance of the difference compared with control is indicated as follows: **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *p* > 0.05 is not indicated. Means ± SEM are shown.

as an indirect anti-apoptotic molecule (Concannon et al., 2003) by inhibiting the proapoptotic proteins caspase-3 (Stetler et al., 2009) and Bax (Havasi et al., 2008). Since both TGF-β₁ and CD40 ligand may be involved in apoptosis during inflammation (Calingasan et al., 2002; Schuster and Kriegstein, 2002), reduced TGFβ1 and CD40 ligand levels induced by Hsp27 may add to the anti-apoptotic effect of Hsp27. Furthermore, in transgenic mice models for AD it has been demonstrated that TGF-β₁ overexpression may lead to Aβ deposits in cerebral blood vessels and meningeal vessels (Wyss-Coray et al., 1997). Moreover, elevated levels of CD40 ligand may lead to endothelial dysfunction and atherothrombosis, whereas inhibition of CD40/CD40 ligand interaction may prevent atherogenesis in animal models (Urbich and Dimmeler, 2004; Chakrabarti et al., 2010; Dominguez-Rodriguez et al., 2010; Yoshioka et al., 2010). Thus, since Hsp27 affects the expression of TGF-β₁ and CD40 ligand, Hsp27 may create a proinflammatory environment, likely both by reducing the production of anti-inflammatory factors and inducing production of proinflammatory cytokines, inhibit apoptotic processes and may alter the vascular micro-environment. However, more study is required to elucidate the exact role of Hsp27 in neurovascular inflammation in AD.

It has been suggested that Aβ itself can induce neuroinflammation (Heneka et al., 2010). In our study, however, Aβ_{1–40}, Aβ_{1–42}, and D-Aβ_{1–40} had no effect on the production of inflam-

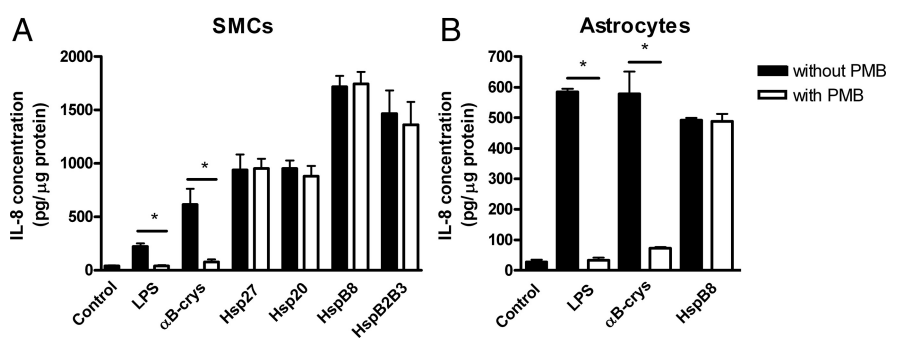


Figure 6. Production of IL-8 by human brain leptomenigeal SMCs (A) and human brain astrocytes (B) after coincubation with PMB. Cultured cells were coincubated with 1 μg/ml LPS or a 12.5 μM concentration of the indicated sHsp with or without 10 μg/ml PMB for 2 d at 37°C. In these experiments, PMB was preincubated with both the LPS and the sHsps for 1 h at 37°C before addition to the cells. Supernatant was collected and IL-8 concentrations were measured using ELISA. PMB efficiently abolished the effects of LPS and αB-crystallin on IL-8 production, but not of the other sHsps. Statistical analysis was performed using an independent sample *t* test. The level of significance of the difference compared with control is indicated as follows: **p* < 0.05; *p* > 0.05 is not indicated. Means ± SEM are shown.

matory factors by SMCs and astrocytes. Previously, we also demonstrated that Aβ did not induce IL-6 production by cerebrovascular cells and astrocytes (Wilhelmus et al., 2009). However, in the same study it was shown that Aβ slightly induced IL-6 production by microglia (Wilhelmus et al., 2009). In addition, in another study dose-dependent increases of IL-8 and MCP-1 production by human brain microglia were observed after exposure to Aβ (Lue et al., 2001). Together, these studies suggest that in the brain, microglial cells may be particularly susceptible to Aβ, resulting in an inflammatory reaction, but that other cerebral cell types, such as vascular cells and astrocytes, do not respond to Aβ in a proinflammatory way, but may be stimulated to do so by sHsps.

In conclusion, Hsp20, HspB8 and HspB2 are associated with (cap)CAA and dyschoric angiopathy in AD brains with severe CAA pathology. In addition, colocalization of these sHsps with ICAM-1 around $\text{A}\beta$ -laden vessels was observed. Furthermore, we demonstrated that Hsp20, HspB8 and HspB2B3 induced the production of inflammatory factors *in vitro*, suggesting that these sHsps might be among the key mediators of the inflammatory reactions associated with (cap)CAA and dyschoric angiopathy. An interesting role for Hsp27 was found, since this sHsp seems to induce a proinflammatory effect by reducing the normal anti-inflammatory effect in human SMC and astrocytes *in vitro*. Furthermore, although it has been suggested that $\text{A}\beta$ itself can induce inflammation (Heneka et al., 2010), we did not observe such an effect in cultured SMCs and astrocytes. The results of this study provide new insights in the inflammatory reactions associated with AD and CAA and, therefore, might provide interesting new targets for therapeutic intervention in the pathogenesis of AD and CAA.

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