CD36, a Class B Scavenger Receptor, Is Expressed on Microglia in Alzheimer's Disease Brains and Can Mediate Production of Reactive Oxygen Species in Response to β -Amyloid Fibrils

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A pathological hallmark of Alzheimer's disease is the senile plaque, composed of β -amyloid fibrils, microglia, astrocytes, and dystrophic neurites. We reported previously that class A scavenger receptors mediate adhesion of microglia and macrophages to β -amyloid fibrils and oxidized low-density lipoprotein (oxLDL)-coated surfaces. We also showed that CD36, a class B scavenger receptor and an oxLDL receptor, promotes H_2O_2 secretion by macrophages adherent to oxLDL-coated surfaces. Whether CD36 is expressed on microglia, and whether it plays a role in secretion of H_2O_2 by microglia interacting with fibrillar β -amyloid is not known. Using fluorescence-activated cell sorting analysis and immunohistochemistry, we found that CD36 is expressed on human fetal microglia, and N9-immortalized mouse microglia. We also found that CD36 is expressed on microglia and on vascular endothelial cells in the brains of Alzheimer's disease patients. Bowes human melanoma cells, which normally do not express CD36, gained the ability to specifically bind to surfaces coated with fibrillar β -amyloid when transfected with a cDNA encoding human CD36, suggesting that CD36 is a receptor for fibrillar β -amyloid. Furthermore, two different monoclonal antibodies to CD36 inhibited H_2O_2 production by N9 microglia and human macrophages adherent to fibrillar β -amyloid by ~50%. Our data identify a role for

CD36 in fibrillar β -amyloid-induced H_2O_2 production by microglia, and imply that CD36 can mediate binding to fibrillar β -amyloid. We propose that similar to their role in the interaction of macrophages with oxLDL, class A scavenger receptors and CD36 play complimentary roles in the interactions of microglia with fibrillar β -amyloid. (*Am J Pathol* 2002, 160:101–112)

Alzheimer's disease (AD) is the most common neurodegenerative disease of adults.¹ It is characterized by the extracellular deposition of insoluble fibrillar β -amyloid protein (fA β) in the brain parenchyma.² Intraparenchymal deposits of fA β are composed of A β peptides 40 to 42 amino acids in length, surrounded by activated microglia, astrocytes, and dystrophic neurites,^{1,2} all of which constitute the senile plaque. Inflammation, initiated by cellular reactions to these intraparenchymal deposits of fA β , is thought to play an important role in the pathogenesis of AD.^{3,4} Microglia, the brain's mononuclear phagocytes, are thought to be the principal cells that mediate this inflammation.^{3–5}

The interaction of neonatal microglia with fA β *in vitro* stimulates these cells to produce proinflammatory and potentially neurotoxic substances such as nitric oxide, tumor necrosis factor- α ,⁵ and reactive oxygen species (ROS).^{6,7} Removal of microglia from cultures containing mixed brain cells and fA β almost totally eliminates the toxic effects of fA β on primary neurons,⁸ suggesting that microglia, and/or substances they produce, mediate the neurotoxic effects of fA β .

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Microglia express class A scavenger receptors (SR-A).⁹ In neonatal microglia these receptors promote endocytosis of $\text{fA}\beta$ in suspension,¹⁰ and adhesion of microglia to $\text{fA}\beta$ -containing surfaces.⁶ SR-A expression is enhanced in microglia in brains of AD patients compared to brains of individuals of similar age who do not have AD,^{11,12} and in the brains of transgenic mice expressing a mutated form of the human amyloid precursor protein (APP23), which develop AD-like pathology.¹³ It is not known whether microglial expression of other scavenger receptors is affected in AD.

Like $\text{fA}\beta$, oxidized low-density lipoprotein (oxLDL) is a ligand for SR-A. OxLDL is also a ligand for CD36.¹⁴ We have shown that SR-A and CD36 play complementary roles in mediating adhesion of human monocyte-derived macrophages to surfaces coated with oxLDL and in secretion of ROS.¹⁵ SR-A participates in adhesion of macrophages to oxLDL-coated surfaces, whereas CD36 signals ROS production but is not required for adhesion to these surfaces. The similarities between interactions of microglia with $\text{fA}\beta$ and of macrophages with oxLDL led us to test expression of CD36 on microglia *in vitro* and in brains of AD patients and to determine whether it plays a role in $\text{fA}\beta$ -induced secretion of ROS by microglia. We report here that CD36 is expressed on microglia *in vitro* and in AD brains and that it cooperates with adhesion-promoting receptors in signaling secretion of ROS by microglia and macrophages *in vitro*.

Materials and Methods

Antibodies

We used several anti-CD36 monoclonal antibodies (mAbs) obtained as follows: SM ϕ (Sigma Chemical Co., St. Louis, MO), NL07 (Pharmingen, San Diego, CA), and FA6-152 (Bioscience International, Kennebunk, ME). Monoclonal antibody anti-CD11b (M1/70) was from Caltag (Burlingame, CA). Rabbit anti-human glial fibrillary acidic protein (GFAP) was a generous gift from Dr. James Goldman, Columbia University, New York, NY.¹⁶ Biotin-SP-conjugated Affinipure F(ab)₂ fragments of rabbit anti-mouse IgG, donkey anti-mouse IgG, and goat anti-human IgG were from Jackson ImmunoResearch (West Grove, PA). Control rat IgG2b was from Zymed (San Francisco, CA), control rabbit anti-serum and monoclonal mouse IgG₁ were from Sigma Chemical Co. Control antibody, MOPC 104E was from Organon Teknika Corp. (Cappel Research Products, Durham, NC). Fluorescein isothiocyanate-labeled goat anti-mouse IgG for fluorescence-activated cell sorting (FACS) analysis was from BD Pharmingen (San Diego, CA).

Fluorescent Dyes and Reagents

Streptavidin-Alexa 568, Amplex Red hydrogen peroxide assay kit, and CyQuant cell proliferation assay kit were from Molecular Probes Inc. (Eugene, OR). Tyramide sig-

nal amplification kit (TSA) for immunohistochemistry was from New England Nuclear (Boston, MA). Amyloid- β peptide 25-35 was from Sigma Chemical Co. or from Bachem Bioscience Inc. (King of Prussia, PA). Amyloid- β peptides 1-42, 42-1, and 35-25 (synthetic peptides with the reverse sequences of $\text{A}\beta$ 1-42 and 25-35, respectively) were from Bachem Bioscience Inc. or American Peptide Company (Sunnyvale, CA). Thrombospondin-1 (TSP-1) and phorbol-12-myristate acetate were from Sigma Chemical Co..

Cells and Transfection

The murine microglial cell line N9, was a generous gift from Dr. P. Ricciardi-Castagnoli (University of Milano, Bicocca, Italy)¹⁷ and was cultured as previously described¹³ in RPMI 1640 medium (Life Technologies, Inc., Long Island, NY) supplemented with 10% heat-inactivated fetal bovine serum and penicillin (100 U/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$). Microglia were isolated from human fetal tissue provided by the Fetal Tissue Bank at Albert Einstein College of Medicine as described.¹⁸ The cells were maintained in Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (100 $\mu\text{g}/\text{ml}$), and 25 mmol/L Hepes. Human monocyte-derived macrophages were prepared as described.¹⁹ Bowes human melanoma cells were obtained from American Type Culture Collection (Rockville, MD), and were maintained in Dulbecco's modified Eagle's medium and 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 $\mu\text{g}/\text{ml}$). The mammalian expression vector for human CD36 (CDM8-CD36) was a generous gift from Dr. Brian Seed (Massachusetts General Hospital, Boston, MA).²⁰ A stable Bowes melanoma cell line expressing human CD36 was generated by co-transfecting CDM8-CD36 with vector pcDNA-neo (Invitrogen, Carlsbad, CA) using the calcium phosphate co-precipitation method and selection in G418 sulfate (Invitrogen) as described previously for SR-A.¹⁹ Mock-transfected cells were generated by co-transfecting CDM8 (not containing CD36 cDNA) and pcDNA-neo in Bowes cells as described above.

Preparation of Fibrillar β -Amyloid

$\text{A}\beta$ 25-35 and $\text{A}\beta$ 35-25 were dissolved in phosphate-buffered saline (PBS) at 1 mg/ml and incubated at 37°C for 1 day.⁶ $\text{A}\beta$ 1-42 and 42-1 were dissolved in either double-distilled H₂O (ddH₂O) alone at 1 mg/ml, or ddH₂O followed by 10 $\times$ PBS to a final concentration of 1 mg/ml and incubated at 37°C for 3 to 4 days as described.⁶ Fibril formation was confirmed by several methods (see Results and Figure 1). Fibrils formed by $\text{A}\beta$ 1-42 were visualized by transmission electron microscopy as described.²¹ Briefly, Formvar-coated nickel grids were incubated on drops of $\text{A}\beta$ 1-42 (1 mg/ml) in PBS for 1 minute and then on drops of 2% phosphotungstic acid for

Figure 1. Characterization of A β peptides. **A** and **B**: Solutions containing A β 42-1 and 1-42 were prepared as described in Materials and Methods and used to coat multislide slides. **A**: Spots coated with A β 42-1 and incubated with Thioflavin S for 1 minute remained unstained implying no fibril formation. **B**: Spots coated with A β 1-42 and incubated with Thioflavin S (Sigma) for 1 minute stained intensely. **C**: A transmission electron micrograph of negatively stained A β 1-42 fibrils (original magnification, $\times 65,000$). **D**: Nonreducing SDS-PAGE of fA β 1-42 and 42-1 peptides stained with Coomassie blue (Sigma). The lane containing fA β 1-42 peptides shows two bands, one at ≈ 4.2 kDa and one at ≈ 21 kDa, whereas the lane containing 42-1 contains a single band at ≈ 4.2 kDa.

1 minute. The samples were photographed in a Philips CM 10 transmission electron microscope at 80kV.

Measurement of ROS and H₂O₂

Because of limitations in availability of primary human microglia, we used nitroblue tetrazolium (NBT) reduction²² to measure ROS production by these cells. Fifty μ l of RPMI containing 25×10^3 microglia and 1 mg/ml NBT (Molecular Probes) and 1 mg/ml bovine serum albumin (BSA) was added to each spot of Multislide slides (Shandon-Lipshaw, Philadelphia, PA) coated with the indicated amount and type of A β peptide and incubated for 30 minutes at 37°C. The medium on each spot was aspirated and the slides were washed three times in methanol

and air-dried. (At this stage, cells can be visualized by light microscopy and the slides can be stored for additional analysis without risk of quenching or loss of signal.) To extract the reduced and precipitated formazan, 30 μ l of 2 mol/L KOH was added to each spot followed by 35 μ l of dimethyl sulfoxide. The entire KOH-dimethyl sulfoxide mixture was transferred to a 96-well plate and the optical density of each sample was read in a multiwell plate reader at 650 nm.

H₂O₂ secretion by macrophages was assayed using 10-acetyl-3,7-dihydroxy-phenoxazine (Amplex Red) and horseradish peroxidase according to the manufacturer's protocol (Molecular Probes), using a Cytofluor II fluorescence plate reader at excitation 530 nm and emission 590 nm. Where indicated, cells were incubated with 20

$\mu\text{g/ml}$ of anti-CD36-mAb (SM ϕ or NLO7), control antibody (MOPC 104E), or 5 $\mu\text{g/ml}$ of TSP-1 in KRBG-A for 30 minutes at room temperature before plating.

Immunofluorescence

Human fetal microglia or N9 cells were plated on coverslips and allowed to adhere at 37°C for 0.5 to 12 hours. The coverslips were then rinsed in Hepes-buffered saline (HBS) (125 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L KH_2PO_4 , 10 mmol/L NaHCO_3 , 20 mmol/L Hepes, 5 mmol/L glucose, 1 mmol/L CaCl_2 , 1 mmol/L MgCl_2), incubated in HBS containing 1% BSA for 30 minutes at 4°C to block nonspecific binding of antibodies. The coverslips were further incubated without fixation at 4°C for 30 minutes with HBS containing 1% BSA and 8 $\mu\text{g/ml}$ mouse monoclonal anti-CD36 antibody FA6-152, or 8 $\mu\text{g/ml}$ of MOPC-31c, an IgG₁ isotype-matched control. The cells were washed with HBS on ice, incubated with a 1:200 dilution of Biotin-SP-conjugated Affinipure F(ab)₂ fragments of rabbit anti-mouse IgG for 30 minutes, fixed in 3.7% formaldehyde for 10 minutes on ice, washed again with HBS, and incubated at room temperature for 30 minutes with a 1:1000 dilution of Streptavidin-Alexa 568 in HBS. Alexa 568 staining was imaged using a Zeiss LSM 410 confocal microscope at the confocal imaging core facility, Department of Anatomy and Cell Biology and the Herbert Irving Comprehensive Cancer Center at Columbia University.

FACS Analysis

N9 microglia were suspended at 10^6 cells/ml in PBS containing 1% BSA and 20 $\mu\text{l/ml}$ phycoerythrin-labeled SM ϕ (PE-SM ϕ) or MOPC (PE-MOPC 104E) antibody, and incubated for 30 minutes at 4°C. The cells were then washed three times in PBS to remove unbound antibodies, and suspended in PBS containing 1% BSA at 10^6 /cells ml. To stain human monocyte-derived macrophages and Bowes melanoma cells transfected with human CD36 cDNA, the cells were incubated with the anti-CD36 antibody FA6-152 (10 $\mu\text{g/ml}$) for 30 minutes on ice in PBS containing 1% BSA, washed three times, followed by another 30-minute incubation with fluorescein isothiocyanate-labeled rabbit anti-mouse IgG. FACS analysis was performed using a Becton Dickinson FACSCalibur as previously described.¹⁹

Immunohistochemistry

Frontal brain samples from patients with AD, Parkinson's disease, amyotrophic lateral sclerosis, and other diseases were obtained from the Brain Bank, Department of Pathology, Columbia University; The Alzheimer's Disease and Schizophrenia Brain Bank; Department of Psychiatry, Mount Sinai School of Medicine; and The Kathleen Price Bryan Brain Bank, Duke University Medical Center. Eight- μm -thick brain sections were cut using a Minotome cryostat (International Equipment Co., Needham Heights, MA), collected on Fisherbrand Superfrost/Plus pre-

cleaned slides, mounted, dried for 2 hours at room temperature, permeabilized by immersion for 10 minutes in -20°C acetone, dried for 2 hours at room temperature, and immediately frozen at -80°C until needed. Staining of CD36 was performed using monoclonal anti-CD36 antibody FA6-152 and direct tyramide signal amplification using New England Nuclear's TSA direct kit (NEL701) to enhance the intensity of the signal achieved by traditional streptavidin-biotin staining. Tissues were stained according to manufacturer's instructions. Briefly, the tissues were blocked for 30 minutes, incubated for 1 hour with 20 $\mu\text{g/ml}$ of FA6-152, or control IgG₁. Slides were then gently washed in PBS, and incubated 30 minutes with a 1:200 dilution of biotin-conjugated donkey or rabbit anti-mouse IgG. Tissues were then washed again in PBS, and incubated with a 1:100 dilution of streptavidin-horseradish peroxidase for 30 minutes then washed with PBS again. The incubation time with tyramide-fluorescein was ~ 6 minutes. To visualize astrocytes, some sections were co-stained with a 1:500 dilution of rabbit polyclonal antibody against human GFAP in HBS for 30 minutes at room temperature as described.¹⁶ To visualize microglia, some sections were co-stained with 1 $\mu\text{g/ml}$ of rat monoclonal antibody M1/70 against mouse and human Mac1 (CD11b/CD18)²³ in HBS for 30 minutes at room temperature. Because microglia also are known to express IgG on their cell surface,²⁴ identification of microglia in some sections was also verified by staining with goat anti-human IgG (data not shown) as described.²⁴ All co-staining antibodies were visualized using a 1:3000 dilution of the appropriate Alexa 594-labeled secondary antibody. Identification of endothelial cells was performed using staining with a 1:500 dilution of fluorescein isothiocyanate-labeled *Ricinus communis* lectin (an established endothelial cell marker)²⁵ obtained from Sigma Chemical Co. as described.²⁶ Slides were mounted in Gel/Mount (Biomedex Corp., Foster City, CA) and coverslips sealed with nail polish before viewing with a Nikon Eclipse E800 microscope with a $\times 60$ objective lens and digital camera (Scientific Instruments, MI) using advanced spot software. Images were assembled in Adobe Photoshop.

Cell Adhesion Assays

Cell adhesion assays were performed as described previously using multispot slides.^{6,19} Where indicated, the Cyquan cell proliferation assay kit (Molecular Probes) was used to measure the number of adherent cells according to the manufacturer's instructions.

Results

Characterization of A β Peptides Used in These Experiments

We confirmed that A β 1-42 peptides prepared as described in Materials and Methods form fibrils *in vitro* by four different methods. First, we used established methods²⁷ to confirm that fA β 1-42 was birefringent under

polarized light whereas A β 42-1 was not. Second, we coated glass slides with fA β 1-42 or A β 42-1, incubated them with Thioflavin S and examined them by fluorescence microscopy as described.²⁸ Glass surfaces coated with A β 42-1 did not fluoresce after staining with Thioflavin S (Figure 1A), whereas those coated with fA β 1-42 did (Figure 1B). Third, using transmission electron microscopy, we found that A β 1-42 formed thin elongated fibrils (Figure 1C) similar to those described by others.²¹ Fourth, analysis of fA β 1-42 by nonreducing SDS-PAGE and Coomassie-blue staining showed two bands, an $\approx$ 4.2-kD band (the monomeric form of the peptide), and a second band of $\approx$ 21 kD (likely to be pentamers of A β 1-42) (Figure 1D). As expected, A β 42-1 peptides ran at $\approx$ 4.2 kD indicating that they did not oligomerize (Figure 1D).

Using more sensitive Western blotting and silver staining analyses other investigators have described up to four different bands under denaturing nonreducing conditions.²¹ The data presented in Figure 1D suggests that the $\approx$ 21-kD species is the dominant oligomer after denaturation of fA β 1-42 under nonreducing conditions. Taken together, the data presented in Figure 1 confirm that A β 1-42 used in the experiments reported here was fibrillar, whereas A β 42-1 was not.

N9 Immortalized Murine Microglia and Human Fetal Microglia Express CD36 on Their Surfaces

Murine microglia express scavenger receptor activity as evidenced by their ability to endocytose DiI-AcLDL²⁴ and adhere to fA β -containing surfaces.⁶ SR-A is expressed by microglia in normal brains¹¹ and in increased amounts by microglia in the brains of patients with AD,^{11,12} and in the brains of transgenic APP23 mice with AD-like pathology.¹³ To test whether CD36 is expressed on microglia, we incubated N9 microglia with anti-CD36 monoclonal antibody PE-SM ϕ or with an isotype-matched PE-labeled control IgM (PE-MOPC 104E) and analyzed the cells by FACS. N9 cells incubated with PE-SM ϕ anti-CD36 were approximately four times more fluorescent than N9 cells incubated with the control antibody indicating that N9 cells express CD36 (Figure 2A). Similarly, human fetal microglia in culture expressed significant scavenger receptor activity as evidenced by their capacity to endocytose DiI-AcLDL (Figure 2B). They also stained with the anti-CD36 antibody FA6-152 (Figure 2, C and D), showing they express CD36 and that the protein is present on their surfaces. Human fetal microglia in culture did not stain with control isotype-matched antibodies (Figure 2, E and F).

Microglia and Macrophages Are Stimulated by fA β to Produce ROS

Primary rat microglia, N9 murine microglia, and human monocyte-derived macrophages produce ROS when incubated with fA β .⁵⁻⁷ To determine whether human microglia also produce ROS in response to fA β , we plated

Figure 2. N9 mouse microglia and cultured human fetal microglia express CD36. **A:** N9 microglia were incubated with phycoerythrin (PE)-labeled monoclonal anti-CD36 antibody clone SM ϕ (PE-SM ϕ) or control antibody (PE-MOPC-104E). Cell-associated fluorescence was measured using FACS analysis as described in Materials and Methods. Mean fluorescence was 197.2 for PE-SM ϕ -labeled cells and 53.9 for PE-MOPC-labeled cells. **B:** Adherent human fetal microglia were incubated with PBS containing 1 mg/ml albumin and 5 μ g/ml DiI-AcLDL for 4 hours at 37°C, washed, fixed in 3.7% formalin, and visualized using a Zeiss fluorescence microscope. **C and D:** Adherent human fetal microglia stained with the anti-CD36 monoclonal antibody FA6-152 as described in Materials and Methods. Surface staining was visualized by confocal microscopy of cells for a single optical section (**C**) and for the entire projection series of 16 sections (**D**). Cells did not stain with an isotype-matched control antibody (**E** and **F**).

these cells on fA β 1-42-containing surfaces and measured their capacity to reduce NBT. Human microglia adherent to surfaces containing fA β 1-42 reduced significantly more NBT than human microglia adherent to surfaces coated with reverse A β 42-1 (control peptide) (Figure 3).

Capacity of fA β -Containing Surfaces to Stimulate Mononuclear Phagocytes to Secrete H₂O₂ Is Correlated with the Cell's Expression of CD36

Fresh human monocytes express low levels of CD36²⁹ and secrete little or no H₂O₂ when plated on oxLDL-containing surfaces,¹⁵ whereas monocytes allowed to differentiate in culture for 3 to 5 days both express CD36²⁹ and secrete H₂O₂ when plated on oxLDL-containing surfaces.¹⁵ To determine whether the capacity of fA β to stimulate H₂O₂ secretion by monocytes and mac-

Figure 3. Fibrillar Aβ 1-42 stimulates cultured human fetal microglia to produce ROS. Human fetal microglia were incubated on multispot slides containing 1, 2.5, or 5 μg of fibrillar Aβ 1-42 (filled squares) or 5 μg of reverse (42-1) nonfibrillar amyloid (filled circle). The amount of ROS produced was measured by NBT reduction as described in Materials and Methods.

rophages is correlated with the pattern of CD36 expression, we plated monocytes and monocyte-derived macrophages, at various stages of maturity, on surfaces coated with collagen IV and fAβ 1-42, fAβ 25-35, or Aβ reverse peptide 35-25. Fresh monocytes secreted very little H₂O₂ in response to fAβ (Figure 4A). As expected,³⁰ fresh monocytes secreted substantial amounts of H₂O₂ in response to zymosan (10 mg/ml) (5.6 ± 1.9 nmol H₂O₂/

2×10^5 cells/hour) or phorbol-12-myristate acetate (100 ng/ml) (11.42 ± 2.1 nmol H₂O₂/2 × 10⁵ cells/hour), confirming that they were capable of H₂O₂ secretion when suitably stimulated.

Monocytes matured in Teflon beakers for 3 to 6 days and then plated on surfaces coated with collagen IV and fAβ 1-42 or fAβ 25-35 secreted significantly more H₂O₂ than fresh monocytes (Figure 4, A and B). In contrast, monocytes matured in Teflon beakers for 3 to 6 days and then plated on surfaces coated with collagen IV and reverse peptides 42-1 or 35-25 secreted little H₂O₂ above background (Figure 4, A and B). Monocytes maintained in Teflon beakers for 7 days or more before plating on surfaces coated with collagen IV and fAβ 1-42 or fAβ 25-35 secreted less and less H₂O₂, according to the length of time beyond 7 days they were maintained in culture before plating (Figure 4A). The capacity of fAβ to promote H₂O₂ secretion by monocyte-derived macrophages paralleled their expression of CD36 (Figure 4C).

Antibodies to CD36 Block ROS Production by Microglia and H₂O₂ Secretion by Macrophages Adherent to fAβ-Coated Surfaces

SR-A and CD36 play complementary roles in mediating macrophage adhesion to oxLDL-coated surfaces and in promoting ROS secretion by macrophages adherent to them.¹⁵ We postulated that SR-A and CD36 play similar roles in ROS production by microglia. To test this hypothesis we incubated N9 cells and human monocyte-derived macrophages with monoclonal anti-CD36 antibodies SMφ or NL07, plated them on fAβ-containing surfaces, and measured their production of oxidants. SMφ and NL07 inhibited ROS production by N9 microglia plated on fAβ by 58% and 52%, respectively (Figure 5A). Similarly, SMφ and NL07 inhibited H₂O₂ secretion by macrophages plated on fAβ 1-42-containing surfaces by 58%

Figure 4. The capacity of monocyte-derived macrophages to produce H₂O₂ parallels their expression of CD36. **A:** Human monocytes/macrophages, cultured for 1 to 14 days in Teflon beakers, were added to wells coated with 2.5 μg of collagen IV overlaid with 2 μg of fibrillar Aβ 1-42 (open triangle), fibrillar Aβ 25-35 (filled square), or Aβ 35-25 (open circle) and incubated for 3 hours. H₂O₂ production was determined using the Molecular Probes Hydrogen Peroxide Assay Kit as described in Materials and Methods. Data presented are mean values ± SEM above background (collagen IV alone) for six experiments, each done in triplicate. **B:** H₂O₂ production by human monocyte-derived macrophages cultured for 5 days in Teflon beakers. Cells were incubated as in **A** above in wells containing collagen IV alone or collagen IV and 2 μg of fAβ 1-42 or 2 μg of Aβ 42-1. Data presented are mean values ± SEM above background (collagen IV alone) for three experiments each done in triplicate. *, Indicates values significantly different ($P < 0.05$) from control (open circles), using Student's test. **C:** Summary of FACS analyses of CD36 expression on the surfaces of human monocytes/macrophages maintained in Teflon beakers and assayed at the times indicated as described in Materials and Methods. H₂O₂ production and CD36 expression were both maximal in cells cultured for 5 days.

Figure 5. CD36 mediates ROS production by microglia and H₂O₂ production by macrophages plated on fAβ-coated surfaces. **A:** N9 microglia (106/ml) were preincubated for 30 minutes with anti-CD36 monoclonal antibody SMφ or NL07 (20 μg/ml) or isotype-matched control antibody MOPC-104E (20 μg/ml). Cells (50,000) were added to each spot of multispot slides coated with fibrillar Aβ 1-42 (black bars). ROS production was measured using the NBT assay as described in Materials and Methods. **B:** Human monocyte-derived macrophages (4 to 6 days in culture) were preincubated with anti-CD36 mAbs (SMφ or NL07, 20 μg/ml) or TSP-1 (5 μg/ml), plated on surfaces coated with 2.5 μg of collagen IV and 2 μg of fibrillar Aβ 1-42 (black bars) or fibrillar Aβ 25-35 (gray bars) and assayed for H₂O₂ secretion as described in Materials and Methods. Data are mean values ± SEM of three experiments done in triplicate. ROS production in response to Aβ 42-1 was similar to background (see Figure 4B, and data not shown).

and 44%, respectively, and on fAβ 25-35-coated surfaces by 43% and 37%, respectively (Figure 5B). Addition of TSP-1, a physiological ligand of CD36³¹ to the medium during plating inhibited H₂O₂ production by 4- to 6-day-old cultured macrophages on fAβ 1-42 or fAβ 25-35-coated surfaces by ~90% (Figure 5B).

Maximal inhibition of H₂O₂ secretion by monoclonal antibodies SMφ and NL07 occurred at 20 μg/ml. At 1 or 5 μg/ml SMφ inhibited H₂O₂ secretion by 5% and 27%, respectively. Maximal inhibition of H₂O₂ secretion by TSP-1 was obtained at 5 μg/ml, whereas 1 and 0.1 μg/ml gave 60% and 17% inhibition, respectively.

Control experiments showed that an isotype-matched control antibody (MOPC 104E) did not inhibit NBT reduction by N9 cells plated on fAβ-coated surfaces, and that SMφ and NL07 did not inhibit zymosan-induced ROS production by these cells (data not shown). Similarly, TSP had no effect on zymosan (10 mg/ml)- or phorbol-12-myristate acetate (100 ng/ml)-induced ROS production by macrophages cultured for 4 to 6 days *in vitro* (data not shown). These results suggest that anti-CD36 antibodies block ROS production by microglia and macrophages by inhibiting CD36's interaction with fAβ, and are consistent with a similar role for TSP-1.

Bowes Melanoma Cells Transfected with CD36 cDNA Adhere to fAβ-Coated Surfaces

To test whether CD36 binds to fAβ 1-42, we transfected Bowes human melanoma cells with a mammalian expression vector for human CD36 and generated a cell line expressing CD36 (Bowes-CD36) (see Materials and Methods). We confirmed expression of CD36 in these cells by FACS analysis (Figure 6A). Bowes-CD36 cells gained the ability to bind to surfaces coated with fAβ 1-42 (Figure 6B), confirming that CD36 promotes binding to fAβ 1-42. Furthermore, binding of Bowes-CD36 to fAβ 1-42 was inhibited by monoclonal antibody FA6-152 but not by control IgG₁ (Figure 6C), confirming that Bowes-CD36 binding to surfaces containing 100 to 500 ng of fAβ 1-42 is CD36 mediated.

Effects of Antibodies to CD36 and of TSP-1 on Adhesion of Microglia and Macrophages to fAβ-Coated Surfaces

We have shown previously that adhesion of microglia to fAβ,⁶ and of macrophages to oxLDL-containing surfac-

Figure 6. Bowes cells expressing CD36 gain the ability to bind to fA β 1-42-coated surfaces. **A:** FACS analysis of Bowes melanoma cells transfected with a mammalian expression vector for human CD36 and stained with a monoclonal antibody FA6-152 to CD36. **B:** Adhesion Bowes cells (2.5×10^5 cells/ml), transfected with a mammalian expression vector for CD36 (CDM8-CD36) or with a control vector (PcDNAneo), and suspended in phosphate buffer containing 5 mmol/L EDTA, to multispot slides coated with 2 μ g of collagen IV and the indicated amount of A β 1-42. **C:** Bowes melanoma cells expressing CD36 were incubated with the indicated concentrations of anti-CD36 monoclonal antibody FA6-152 or an isotype-matched IgG₁ control and plated on multispot slides coated with 2 μ g of collagen IV and 500 ng of A β 1-42.

es,¹⁵ is required for ROS/H₂O₂ production. We have also shown that anti-CD36 antibodies block ROS production by macrophages adherent to surfaces coated with oxLDL but have no significant effect on adhesion to these surfaces, suggesting that CD36 is not required for macrophage adhesion. In parallel studies, we tested whether anti-CD36 antibodies SM ϕ or NL07, control antibody MOPC 104E, or the CD36 ligand TSP-1³¹ affected adhesion of microglia and macrophages to surfaces coated with collagen IV and fA β 1-42 or fA β 25-35. None of the antibodies (data not shown) inhibited significantly adhesion of N9 microglia or of macrophages to surfaces containing fA β 1-42 or 25-35. TSP-1 reduced adhesion of macrophages to surfaces coated with fA β 1-42 or fA β 25-35 by ~28% and ~21% respectively. Although this reduction in macrophage adhesion is significant, it is far less than the 90% reduction in H₂O₂ secretion effected by TSP-1 (Figure 5B). These results imply that CD36 functions are blocked more effectively by ligand-receptor interactions than antibody-receptor interactions and/or that receptors for TSP-1 and/or fA β other than CD36³² may contribute to adhesion of macrophages to fA β -containing matrices and to signaling ROS secretion.

CD36 Is Expressed on Microglia in Brains of Patients with Alzheimer's Disease

To confirm that microglia express CD36 *in vivo* we stained frozen sections of unfixed brains from 11 patients using the anti-CD36 antibody FA6-152. These included five patients with AD, one patient with Parkinson's disease, three patients with amyotrophic lateral sclerosis, and two patients without clinical signs of dementia. The immunostained sections shown in Figure 7 are derived from the brain of a patient with AD. The staining is representative of stained frozen sections from all 11 brains studied. In all sections, cells with the appearance of microglia (Figure 7, a and d) reacted with the anti-CD36 antibody FA6-152. CD36-expressing cells stained with antibodies against Mac-1 (CD11b/CD18) (Figure 7b), a microglial mark-

er,^{33,34} but not with antibodies against GFAP (Figure 7e), an astrocyte marker.^{16,35} An overlay showing co-localization of anti-CD36 and anti-Mac-1 staining is shown in Figure 7c, and an overlay showing non-co-localization of anti-CD36 and anti-GFAP staining in Figure 7f. CD36 was expressed to variable extents on cells with microglial morphology in all 11 brains tested (data not shown). Because of the limited number of specimens examined, and the difficulty of detecting modest differences in protein expression by immunohistochemistry, we cannot determine whether CD36 expression is enhanced in microglia in brains of patients with AD compared to microglia in brains from normal aged people or from people with other neurological diseases. In addition to microglia, structures resembling vessels (Figure 7g) also stained with anti-CD36 antibodies. To confirm that these structures are blood vessels we co-stained with fluorescein isothiocyanate-labeled *Ricinus communis* lectin, an endothelial cell marker. As seen in Figure 7, j to l, staining with CD36 correlated with staining for endothelial cells, indicating that in addition to microglia, brain endothelial cells also express CD36.

Discussion

It is now well documented that interactions between microglia and fA β stimulate human microglia to produce ROS and tumor necrosis factor- α , and rodent microglia to produce ROS, nitric oxide, and tumor necrosis factor- α .⁶⁻⁸ These and other findings have led us, and a number of other investigators to suggest that these microglial secretory products contribute to the progression of AD. The studies reported here provide further support for this concept and demonstrate for the first time that human microglia express CD36 *in vitro* and *in vivo*, and that interactions between CD36 and fA β signal ROS production by microglia and macrophages. Consistent with this suggestion, macrophage colony-stimulating factor, a known stimulator of macrophage CD36 expression³⁶ is

Figure 7. CD36 is expressed by microglia and endothelial cells in AD brains. Staining of frontal cortex from 13 patients was performed using New England Nuclear's Tyramide Signal Amplification kit as described under Materials and Methods. Each row represents immunofluorescent staining of a single section from an AD brain and is typical for all sections examined in this study. Staining with anti-CD36 antibody is shown in **a, d, g, and j**, anti-MAC-1 (CD11b) antibody in **b** and **h**, anti-GFAP in **e**, and staining with Ricin in **k**. Overlays of staining in each row are shown in **c, f, i, and l**. Cells lining brain vasculature (likely endothelial cells) (**k**) express CD36 (**j** and **l**) but not the microglial marker Mac-1 (**g, h, and i**).

also expressed in brains of mice with AD-like pathology.³⁷ Macrophage colony-stimulating factor-M enhances the proinflammatory effects of $\text{fA}\beta$ on microglia,³⁸ possibly by enhancing their expression of CD36.

The mechanism(s) by which $\text{fA}\beta$ stimulates CD36 to signal H_2O_2 secretion is unknown. Anti-CD36 antibodies such as SM ϕ and NL07 and soluble CD36 ligands in

suspension such as oxLDL, AcLDL, and TSP-1¹⁵ do not stimulate macrophages to secrete H_2O_2 . However, anti-CD36 antibodies stimulate H_2O_2 secretion by macrophages when cross-linked by a secondary antibody,³⁹ and surfaces containing immobilized CD36 ligands such as oxLDL, acetyl LDL, and TSP-1 stimulate macrophages to adhere and secrete H_2O_2 (and data not shown).¹⁵ Thus

Figure 8. Cooperation between scavenger receptors and CD36 mediates microglial release of ROS. Diagram summarizing proposed effects of CD36 on ROS production by microglia adherent to surfaces coated with A β fibrils. SR-A and other scavenger receptors mediate microglial adhesion to A β -coated surfaces. Engagement of CD36 by substrate bound fA β signals ROS and H₂O₂ production. Pre-exposure of cells to antibodies against CD36 or the CD36 ligand TSP-1 blocks interactions between CD36 and fA β resulting in inhibition of ROS and H₂O₂ production (see Figures 2 and 4).

oligomerization of CD36 by fA β , a multivalent ligand, may be required for CD36 to signal macrophage H₂O₂ secretion.

In addition to ROS production, CD36 can mediate adhesion to fA β -coated surfaces. Bowes melanoma cells, which normally do not bind to surfaces coated with 100 to 500 ng of fA β in the presence of ethylenediaminetetraacetic acid, gain the ability to adhere to such surfaces when transfected with CD36. Monoclonal anti-CD36 antibodies block this process suggesting that CD36 is a receptor for fA β . But CD36-blocking antibodies do not affect the adhesion of microglia and macrophages to fA β -coated surfaces. This finding suggests that microglia and macrophages express other receptors, in addition to CD36, that can mediate adhesion to fA β . This result is also consistent with our previous finding that antibodies to SR-A block most of the adhesion of neonatal microglia to fA β -coated surfaces⁶ and with findings of Husemann and colleagues⁴⁰ and Chung and colleagues⁴¹ that macrophages and microglia from SRA-KO mice endocytose and/or bind ~60% less fA β than cells from wild-type mice. We propose that multiple scavenger- and adhesion-promoting receptors cooperate in mediating adhesion of microglia to fA β in senile plaques.

CD36 also may play a role in Alzheimer's angiopathy. Endothelial cells of the cerebral vasculature, the site of fA β deposition in Alzheimer's angiopathy,^{42,43} also express CD36.⁴⁴ Malaria-infected erythrocytes interact with CD36 on endothelial cells in the cerebral microvasculature and cause disruption of the blood brain barrier.⁴⁵ Fibrillar A β also causes disruption of the blood brain barrier.⁴⁶ We suggest that interaction of CD36 with fA β deposited in the subendothelial space may contribute to the increase in permeability of cerebral microvessels observed in AD.⁴⁷

There are conflicting reports about whether mice genetically deficient in SR-A and maintained on an athero-

sclerosis-promoting diet develop larger or smaller vascular lesions than wild-type mice.^{48,49} Similarly, there are discrepancies between *in vitro* studies done with microglia from SR-A KO mice and *in vivo* studies done with transgenic PD-APP mice genetically deficient in SR-A. Although neonatal microglia from SR-A-KO mice endocytose ~60% less fA β ,^{40,41} Huang and colleagues⁵⁰ reported that transgenic PD-APP mice genetically deficient in SR-A show no obvious change in their AD-like pathology compared to transgenic PD-APP mice expressing SRA. PD-APP mice may not be the best model to study the role of microglia in AD because they exhibit a weaker microglial response compared to other transgenic APP mouse models of AD and to AD patients.^{13,51,52} Nonetheless, the findings reported by Huang and colleagues⁵⁰ suggest that other receptors in addition to SR-A may be involved in mediating the interactions of microglia with fA β in mice. Mice genetically deficient in CD36 and maintained on a high-fat diet have 75% fewer atherosclerotic lesions than wild-type mice maintained on the same diet.⁵³ We are currently investigating whether transgenic APP mice genetically deficient in CD36 have altered AD-like pathology when compared to transgenic APP mice expressing CD36.

The roles we propose for microglial scavenger receptors in AD mirror in several respects the roles reported for macrophage scavenger receptors in atherosclerosis. Oxidation of LDL in the intima of the arterial wall is one of the first events in the development of atheromata and is thought to be a major factor in the initiation and progression of atherosclerosis. SR-A mediates the adhesion of monocytes and macrophages to surfaces containing oxLDL and probably contributes to the localization of these cells at sites of oxLDL deposition¹⁵ and basement membrane modification.¹⁹ Adhesion of monocytes/macrophages to oxLDL-containing surfaces facilitates the interaction of CD36 with oxLDL,¹⁵ and signals them to secrete ROS,¹⁵ and other proinflammatory substances. We suggest that a similar inflammatory cascade is initiated when microglia interact with fA β deposits in the brain parenchyma and perivascular spaces. Microglia adhere to fA β and possibly other components of the senile plaque via SR-A,⁶ CD36 (Figure 6), and other adhesion promoting receptors. This signals microglia to secrete ROS (Figures 3, 5, and 8), and possibly other neurotoxic substances. We propose that SR-A, CD36, and possibly other adhesion and/or scavenger receptors cooperate in mediating binding to A β fibrils in senile plaques, thereby initiating a signaling cascade that leads to production of ROS and other proinflammatory and neurotoxic substances. CD36 may therefore be a key microglial receptor contributing to fA β -induced neuronal damage in AD.

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