Expression of Scavenger Receptors in Glial Cells
COMPARING THE ADHESION OF ASTROCYTES AND MICROGLIA FROM NEONATAL RATS TO SURFACE-BOUND β-AMYLOID*

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Astrocytes and microglia associate with amyloid plaques, a pathological hallmark of Alzheimer disease. Microglia are activated by and can phagocytose β-amyloid (Aβ). Scavenger receptors (SRs) are among the receptors mediating the uptake of fibrillar Aβ in vitro. However, little is known about the function of the astrocytes surrounding the plaques or the nature of their interaction with Aβ. It is unknown whether glial cells bind to nonfibrillar Aβ and if binding of astrocytes to Aβ depends on the same scavenger receptors described for microglia. We determined the binding of glia to Aβ by an adhesion assay and evaluated the presence of scavenger receptors in glial cells by immunocytochemistry, immunohistochemistry of brain sections, and immunoblot. We found that astrocytes and microglia from neonatal rats adhered in a concentration-dependent manner to surfaces coated with fibrillar Aβ or nonfibrillar Aβ. Fucoidan and poly(I), known ligands for SR-type A, inhibited adhesion of microglia and astrocytes to Aβ and also inhibited Aβ phagocytosis. In contrast, a ligand for SR-type B like low density lipoprotein, did not compete glial adhesion to Aβ. Microglia presented immunodetectable SR-BI, SR-AI/AII, RAGE, and SR-MARCO (macrophage receptor with collagenous structure, a member of the SR-A family). Astrocytes presented SR-BI and SR-MARCO. To our knowledge, this is the first description of the presence of SR-MARCO in astrocytes. Our results indicate that both microglia and astrocytes adhere to fibrillar and nonfibrillar Aβ. Adhesion was mediated by a fucoidan-sensitive receptor. We propose that SR-MARCO could be the scavenger receptor responsible for the adhesion of astrocytes and microglia to Aβ.

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The abbreviations used are: Aβ, fibrillar β-amyloid; Aβ, β-amyloid; nAβ, nonfibrillar Aβ; SR, scavenger receptor; RAGE, receptor for advanced glycation end products (RAGE); LDL, low density lipoprotein; AD, Alzheimer disease; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; GFAP, glial fibrillary acidic protein; NAMx, nonadherent matrix; PBS, phosphate-buffered saline; Tricine, N-[2-hydroxyethyl]-1,1-bis[hydroxymethyl]ethyglycine; oxLDL, oxidized LDL; DMEM, Dulbecco’s modified Eagle’s medium.

Advanced glycation end products (RAGE) (4), low density lipoprotein receptor-related protein (5), and mannose receptor (6). Scavenger receptors (SRs) participate in the binding and uptake of many structurally unrelated substances, including fAβ, thrombospondin-1, anionic polysaccharides, polynucleotides, chemically modified proteins (e.g. oxidized LDL, acetylated LDL, glycated collagen, and maleylated albumin), apoptotic cells, and bacteria (2, 7, 8).

Scavenger receptors have been implicated in microglial binding to Aβ. However, their participation has not been adequately characterized in astrocytes. Astrocytes serve important metabolic and supportive functions in the central nervous system. Under physiological conditions, they maintain the functional integrity of synapses and produce some of the glycoproteins of the extracellular matrix (9). They regulate glutamate and water balance and have a role in the maintenance of the blood-brain barrier tight junctions. Astrocytes express several chemokines, cytokines, and their receptors and have phagocytic and proteolytic activity (10–12). Whether normal adult astrocytes can perform phagocytosis remains an unresolved issue. However, there is some evidence that under certain conditions astrocytes are capable of phagocytosis (13, 14). In contrast to microglia, only SR-BI seems to be expressed by astrocytes from adult mouse and human brains (15), although there are reports suggesting that astrocytes also express the mannose receptor (16). Under pathological conditions, like injury of the nervous system, the activation of astrocytes has been associated with changes in the expression of cytoplasmatic proteins, surface proteins, and soluble factors, such as chemokines (10, 12).

Alzheimer disease (AD) is a degenerative disorder characterized by selective neuronal loss and the presence of two types of protein aggregates, amyloid plaques, and neurofibrillary tangles (17, 18, 19, 20). Amyloid plaques are complex deposits composed mainly of fAβ (21, 22) closely associated with microglia and astrocytes (23–25). fAβ is neurotoxic in vitro (26). In AD brains, microglia are activated at the site of deposition of Aβ (27), and astrocytes are most closely associated with plaques with a dense amyloid core and activated microglia (28, 29). Activated astrocytes surrounding amyloid plaques or exposed to fAβ in vitro increase the expression of inducible nitric-oxide synthase (30) and also secrete proinflammatory molecules such as interleukin-1β, MCP-1, RANTES, etc. (10, 31). It is unknown which receptor mediates the activation of astrocytes by Aβ.

SR-MARCO is a newly described SR. It was initially characterized as a bacteria binding receptor. However, it also binds modified LDL; therefore, it is considered a member of the SR-A family (32, 33). There is little information regarding the expression of SR-MARCO in the central nervous system, although its presence in microglial cells was recently described (34, 35).
Recent studies showed that astrocytes from adult mice bind αβ by receptors sharing characteristics with SRs. However, the results suggested that SR-BI did not bind αβ (36). Furthermore, the binding of microglial cells and astrocytes to fibrillar versus nonfibrillar αβ has been poorly examined. Here we report that astrocytes from neonatal rats adhered to surfaces coated with αβ and nonfibrillar αβ (nαβ). Binding to αβ was almost completely inhibited by fucoidan and polycl(I). Furthermore, we found that SR-MARCO was present both in astrocytes and microglia, and its expression by astrocytes was confirmed on sections of adult rat brain. We propose that SR-MARCO could be responsible for the interaction of astrocytes and microglia with αβ.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents—**Fucoidan, poly(C), poly(I), poly-L-lysine, bovine serum albumin (BSA), lidocaine, and trypsin EDTA were purchased from Sigma. Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, and penicillin/streptomycin were obtained from Invitrogen. Tissue culture dishes and plastic ware were obtained from Falcon (Franklin Lakes, NJ) and Nalgene Nunc (Rochester, NY). The cytotoxicity detection kit was obtained from Roche (Roche Applied Science). Other reagents were purchased from Sigma or Merck. The αβ(1–42) was a generous gift from Dr. Heinz Dobeli (Hoffmann-La Roche, Switzerland).

**Glial Primary Cultures—**Astrocytes and microglial cultures were prepared from brains of 1–2-day-old neonatal rats (37). Newborns were anesthetized with ether and decapitated. Cortices were minced and incubated with 0.25% trypsin-EDTA in Hank’s solution (0.4 g/liter KCl, 0.06 g/liter KH2PO4, 0.048 g/liter Na2HPO4, 8 g/liter NaCl, 1 g/liter d-glucose, and 3.5 g/liter NaHCO3) for 15 min and mechanically dissociated. Cells were seeded in 75-cm2 culture flasks coated with poly-L-lysine (one brain cortex in 100 ml DMEM/F-12 or PBS. The number of adherent cells was determined by the LDH assay. Each condition was tested in three independent experiments. Cells were washed with PBS, and the number of cells was determined by the LDH assay. Wells were washed with PBS, and the number of cells was determined by the LDH assay.

**Cell Adhesion Assay—**A 96-well tissue culture plate was coated with a nonadherent matrix (NAMx), collagen IV for microglia or BSA for astrocytes. Wells were treated with 30 μl of 50 μg/ml collagen IV or 50 μl of 1 mg/ml BSA in distilled water at 37 °C for 1 h, washed once with distilled water, and air-dried under sterile conditions. Then β-amyloid fibrils (0.1–2.5 μg) were added and air-dried. For the adhesion assay, 100 μl of DMEM/F-12 plus 1 mg/ml BSA in a 96-well plate and placed in an incubator with 5% CO2 at 37 °C for 1 h. The plates were then washed three times with DMEM/F-12 or PBS. The number of adherent cells was determined by the LDH assay. 3–7 independent experiments were carried out in quadruplicate.

**Ligand Competition Assay—**Glial cells (5 × 104 cells/ml) were preincubated in suspension in DMEM/F-12 plus 1 mg/ml BSA with different known ligands for scavenger receptors for 30 min: 200 μg/ml fucoidan (SR-A ligand), 100 μg/ml LDL (SR-BI ligand), 100 μg/ml oxidized LDL (oxLDL, SR-A and SR-BI ligand), 200 μg/ml Mel-BSA (SR-A and SR-B ligand), 200 μg/ml poly(I) (SR-A ligand), 200 μg/ml poly(C) (negative control for poly(I) adhesion assays). After preincubation, the cells were plated on αβ-coated wells and incubated at 37 °C for 1 h. Because preincubation with the competing ligands may result in the endocytosis of cell surface proteins, including receptor complexes, cells were preincubated with competing ligands also at 4 and 20 °C to block endocytosis. Wells were washed with PBS, and the number of cells was determined using the LDH assay. Each condition was tested in three independent experiments in quadruplicate.

**Binding Inhibition Assay: Immune Neutralization of SR-MARCO—**Astrocytes: Alexa 488-immunoglobulin (MCA 1489, 1:100), Alexa 568-immunoglobulin (MCA 1649, 1:100), Alexa 546-immunoglobulin (MCA 1322), or Alexa 488-anti-rabbit IgG (clone 2F8; Serotec), polyclonal goat anti-RAGE (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or lectin Alexa-488 (1:200; Molecular Probes, Inc., Eugene, OR). For the identification of the novel receptor of SR-MARCO in astrocytes, two separate antibodies from independent sources were tested: rat anti-mouse MARCO (MCA 1849, 1:100; Serotec) and monoclonal mouse anti-MARCO (1:100; BD Biosciences). Because reports suggested a possible cross-reactivity of the monoclonal mouse anti-MARCO with tubulin, live cells were stained in order to restrict labeling to extracellular epitopes. Excess for the samples labeled with lectin, coverslips were washed in PBS and incubated with the corresponding secondary antibody (all from Molecular Probes) diluted 1:100 in blocking solution: Alexa 488-anti-rabbit, Alexa 488-anti-mouse, Alexa 568-anti-rabbit, Alexa 546-anti-goat, and Alexa 555-anti-goat, at room temperature for 2–4 h. Coverslips were washed in PBS and water. Finally, samples were mounted in fluorescein mounting medium (Dako). As a standard practice, to discard the possibility of nonspecific binding, control assays where the primary antibody was omitted were run in parallel.
plated on glass coverslips as described above. Rat anti-mouse MARCO (MCA 1849; 1:100; Serotec) or monoclonal mouse anti-MARCO (1:100; BD Biosciences) was added to the culture media and incubated at 37 °C for 2 h. Cells were washed with 1× PBS, 0.1 mM Ca2+/Mg2+ and then fixed with methanol at −20 °C for 30 min. Cells were blocked with 10% goat serum in PBS and then incubated with rabbit anti-GFAP (1:200) or Alexa 488-lectin (1:200) at 4 °C overnight. After they were washed, samples were incubated with the secondary antibody: Alexa 546-anti-mouse or Alexa 546-anti-rat (1:100; Molecular Probes) for MARCO and FITC-anti-rabbit (1:100; Molecular Probes) for GFAP at room temperature for 2 h. The samples were rinsed with PBS and mounted in mounting medium (Dako).

Immunohistochemical Staining of Brain Sections—Brains from juvenile (150 g) and adult (250 g) rats were obtained after fixation through cardiac perfusion with 4% p-formaldehyde in phosphate-buffered saline. Those brains were post-fixed in 4% p-formaldehyde for 24 h, and the brains of neonatal rats were fixed for 12 h and then transferred to 15% sucrose at 4 °C. Cryosections were prepared at 8–20 μm and stored at −20 °C. Immunohistochemistry was performed on attached and free floating sections using the anti-SR-MARCO MCA 1849 antibody. Double staining was carried out using an antibody against GFAP to identify astrocytes. Sections were incubated in blocking solution (PBS supplemented with 10% goat serum, 0.2% Triton X-100, 0.02% azide) for 1 h. Immunodetection was performed by incubation in primary antibodies diluted 1:50 in blocking solution at 4 °C for 48 h. Sections were washed four times with PBS, incubated with secondary antibodies diluted 1:100 in blocking solution at room temperature for 2 h, and washed again with PBS. Specific staining was absent if primary antiserum was omitted.

Immunoblot Analysis—Gliaal cell cultures were prepared as indicated in the description of cell culture methods. Cells were harvested and lysed in an ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, and protease inhibitors). Protein concentration of cell lysates was determined by the BCA method. The lysates (same amount of protein for the different samples of each independent experiment) were dissolved in sample buffer (80 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 2% 2-mercaptoethanol, 0.02% Comassie Blue G250) and were heated in a boiling water bath for 5 min. SDS-PAGE was performed with 7.5 and 10% (w/v) polyacrylamide gels and transferred to a nitrocellulose membrane (Amersham Biosciences). After electrophoretic transfer, the membrane was treated with the blocking buffer (PBS plus 0.05% Tween 20, 10% goat serum). The membrane was then incubated with the primary antibody in blocking buffer: anti-SR-A antibody (1:500, Clone 2F8; Serotec), anti-SR-B antibody (1:2000; Novus Biologicals), anti-sr-MARCO antibodies (1:1000, MCA 1849; Serotec) and 1:1000, clone 22 (BD Biosciences) or anti-RAGE antibody (1:500; N-16; Santa Cruz Biotechnology). The primary antibody was rinsed off the membrane was incubated with horseradish peroxidase-labeled goat anti-rabbit IgG, horseradish peroxidase-labeled goat anti-rat IgG, horseradish peroxidase-labeled goat anti-mouse IgG, or horse-radish peroxidase-labeled donkey anti-goat IgG in blocking buffer as the secondary antibody, depending on the primary antibody previously used. Signals were detected by enhanced chemiluminescence (Amer-sham Biosciences) in accordance with the manufacturer’s instructions. The molecular mass was estimated with a molecular mass marker kit (Invitrogen). Lysates obtained from adrenal gland (SR-B), liver (SR-A and SR-MARCO), and lung (RAGE) were used as positive controls.

Aβ Removal Assays—Glass slides in 24-wells plates were overlaid with poly-L-lysine and later a central spot of Cy3-Aβ or plain nAβ until dried, washed with sterile water, and air-dried. 2 × 104 astrocytes were plated on the coated glass slides and incubated for up to 2 days at 37 °C. For the experiments with Cy3-Aβ, cells were fixed in 5% formalin at room temperature for 20 min, and nuclei were stained with 0.1 μg/ml Hoechst 33258 (S8283; Sigma). When unlabeled nAβ was used, cells were fixed for 30 min in 4% formaldehyde in PBS and permeated with PBS plus 0.2% Triton X-100. Cells were blocked with 5% goat serum in PBS and then incubated with a rabbit polyclonal antibody against glial fibrillary acidic protein (GFAP; 1:200; Dako) as identity marker for astrocytes and a monoclonal antibody anti-Aβ amyloid (BAS-18; 1:200) at 4 °C overnight and the corresponding secondary antibody (Molecular Probes): Alexa-546-conjugated anti-mouse (1:100) and Alexa-488-conjugated anti-rabbit (1:100) at room temperature for 2–4 h. Nuclei were also stained with 0.1 μg/ml Hoechst 33258.

Statistical Analysis—The statistical significance of the differences in adhesion between the different substrate and competition assays was analyzed with the Mann-Whitney U test. Statistical significance was established for p < 0.05.

RESULTS

The purity of the cells for the adhesion assays was evaluated by immunocytochemistry with cell identity markers. Microglial cells were over 99% pure, and astrocytes contained less than 5% of contaminating microglia.

Binding of Astrocytes and Microglia of Neonatal Rat to Different Matrices—We compared the adhesion of astrocytes and microglia with different matrices (Fig. 1A). After 1 h of assay, the adhesion of astrocytes was maximal on poly-L-lysine and minimal on BSA (6 ± 2% cells) (Fig. 1A). Microglial adhesion to poly-L-lysine, plastic, and BSA was 100 ± 3, 116 ± 3, and 91 ± 4%, respectively (Fig. 1A). In contrast, they adhered poorly to gelatin (18 ± 6%) and collagen type IV (29.1 ± 8.6%), as previously reported (46).

To determine the adhesion kinetic of astrocytes to different matrices, we plated cells on poly-L-lysine, collagen, and BSA for different times (Fig. 1B). After the first hour, most astrocytes adhered to poly-L-lysine. Adhesion to collagen was poor, and cells did not adhere to BSA during the 24 h of the experiment. Considering these results, BSA was used as the NAMx for astrocytes, and collagen type IV was used for microglia.

Characterization of nAβ and nAβ—Aβ aggregation resulted in the formation of soluble nAβ and insoluble Aβ fibrils. The Western blot analysis (Fig. 2A) of nAβ with nonreducing conditions showed two well-defined bands: a 4.1-kDa band corresponding to the monomer (33%) and a second band of 8.4 kDa corresponding to the dimeric form (60%). A third weak band
of ~12 kDa and a fourth weak band of ~36 kDa (likely to be oligomers) were only observed when 1 μg of nfAβ was loaded. Under reducing conditions, two bands were observed at 4.1 kDa (32 and 38%) and at 8.4 kDa (68 and 60%), for 0.5 and 1 μg of nfAβ, respectively. A third weak band of ~12 kDa was only observed on 1 μg of nfAβ. fAβ under reducing conditions also showed four bands, of 4.1, 8.4, ~12, and ~36 kDa, that correspond to 43, 54, 3, and 1%, respectively (Fig. 2A). The immunoblot of fAβ under nonreducing conditions was not included because the fibers do not enter the electrophoresis gel.

Congo Red Binding Assays—To rule out the aggregation of nfAβ when the Aβ matrix was prepared, kinetic experiments with an established probe for fibrillar Aβ were performed. The detection of amyloid fibrils in the matrix was followed over time up to 24 h (Fig. 2B). Whereas detection of fAβ nearly doubled during the 24 h of observation, detection by Congo red in the nfAβ matrix did not change over time, being only 25% higher than the measurement for the negative control with the non-adhesive matrix. Hence, the results obtained are in agreement with nfAβ remaining in a nonfibrillar state.

Astrocytes and microglia of neonatal rats showed similar adhesion to surfaces coated with nfAβ and fAβ—Microglia and astrocytes were attached to nonadherent matrix coated with 1 μg of fAβ or nfAβ after 1 h (Fig. 3A). For quantification, adhesion was compared with the adhesion of glial cells to poly-lysine. Astrocyte adhesion to surfaces coated with 1 μg of fAβ or nfAβ was 85.4 ± 6% and 80.1 ± 3.2% (compared with less than 10% for NAMx, p < 0.001) whether microglial cell adhesion was 72.7 ± 5.3 and 82.8 ± 3.4% (p < 0.01 compared with NAMx), respectively.

Adhesion of glial cells to Aβ was concentration-dependent (Fig. 3B). Although the maximal adhesion was at 2.5 μg, the highest amount of Aβ tested, adhesion reached its plateau at ~1 μg of Aβ, for both the fibrillar (fAβ) and nonfibrillar (nfAβ) forms. Maximal adhesion of astrocytes was 103.7 ± 3.7% (nfAβ) and 85.4 ± 6% (fAβ); 50% of that adhesion was observed at 0.4 μg Aβ (~50% of plated astrocytes). Adhesion of microglia to Aβ was similar (Fig. 3B), with the maximal adhesion between 94 ± 4.3% (nfAβ) and 72.7 ± 5.3% (fAβ) and 50% of that adhesion observed at 0.2 μg of Aβ (between 35 and 45% of plated microglia, respectively).

Fucoidan, Poly(I), and oxLDL Block Adhesion of Newborn Rat Astrocytes to nfAβ and fAβ—Different SR ligands, such as fucoidan, a polysaccharide previously thought to specifically inhibit SR class A type I and II (1), and oxLDL and Mel-BSA (chemically modified lipoprotein and protein, respectively), binding all SRs (47), were tested. In general, most of the ligands that were capable of inhibiting glial adhesion were better competitors for the binding to nfAβ than for fAβ. Fucoidan inhibited microglial adhesion to fAβ by 37.5-fold and inhibited adhesion to nfAβ by 32-fold (p < 0.001). By comparison, fu-
Adhesion of astrocytes and microglia of newborn rats to fAβ and nAβ: Competition assays with SR ligands at low temperature to inhibit endocytosis

Gliaal cells were preincubated with the competing ligands, with gentle agitation at 37 °C, for 30 min: 100 μg/ml oxLDL, 100 μg/ml LDL, 200 μg/ml Mel-BSA, 200 μg/ml poly(I), 200 μg/ml poly(C), or 200 μg/ml fucoidan. 100% adhesion corresponds to 1.24 × 10^4 microglia or 3.03 × 10^4 astrocytes. Values represent the mean ± S.E. of three independent experiments, performed in quadruplicate.

| Nonadherent matrix coating | Competing ligand | Adherent cells (percentage of control) |
|---------------------------|-----------------|---------------------------------------|
|                          |                 | Astrocyes                              | Microglia                            |
| fAβ                      | None            | 100 ± 3.5                              | 99.7 ± 7.0                           |
|                          | LDL             | 96.4 ± 6.6                             | 92.1 ± 6.1                           |
|                          | oxLDL           | 16.9 ± 2.4                             | 16.1 ± 4.3                           |
|                          | Fucoidan        | 17.0 ± 1.8                             | 2.7 ± 1.1                            |
|                          | Mel-BSA         | 28.5 ± 2.9                             | ND                                   |
|                          | Poly(I)         | 13.5 ± 3.7                             | ND                                   |
|                          | Poly(C)         | 86.2 ± 10.3                            | ND                                   |
| nAβ                      | None            | 100 ± 2.0                              | 100 ± 6.9                            |
|                          | LDL             | 75.0 ± 3.5                             | 91.6 ± 6.8                           |
|                          | oxLDL           | 3.7 ± 1.0                              | 3.7 ± 3.2                            |
|                          | Fucoidan        | 6.2 ± 3.0                              | 3.1 ± 1.6                            |
|                          | Mel-BSA         | 7.5 ± 2.5                              | ND                                   |
|                          | Poly(I)         | 11.0 ± 4.4                             | ND                                   |
|                          | Poly(C)         | 69.0 ± 3.8                             | ND                                   |

* a p < 0.001, Mann-Whitney U test.
* b ND, not determined.
* c p < 0.01, Mann-Whitney U test.

coidan inhibited the adhesion of astrocytes to fAβ by 5.9-fold and to nAβ by 16-fold (Table I; p < 0.001). Similar results were obtained with poly(I) (also a ligand for type-A SR), which inhibited the adhesion of astrocytes to fAβ by 7.4-fold and to nAβ by 9-fold (p < 0.01). poly(C) (negative control) did not inhibit the adhesion to fAβ and only had a mild effect on the adhesion to nAβ. Native LDL did not inhibit the adhesion of microglia to fAβ or nAβ. Similarly, LDL did not significantly affect astrocyte adhesion to fAβ and only marginally (1.3-fold) reduced the binding of astrocytes to surfaces coated with nAβ (Table I). In contrast to its inhibitory effect on the binding of neonatal rat microglia and astrocytes to nAβ (a 27-fold reduction in binding, p < 0.001), oxLDL only reduced partially (6-fold, p < 0.01) the adhesion of microglia and astrocytes to fAβ (Table I). Similar results were obtained when cells were preincubated with competing ligands at 20 or 4 °C (Table II); poly(I) inhibited the adhesion of astrocytes to fAβ by 7.4-fold at 37 °C, 9.5-fold at 4 °C, and 15-fold at 20 °C (p < 0.001 compared with the adhesion in the absence of competing ligand).

Glia of Newborn Rats Exposed Scavenger Receptors and RAGE

—The presence of scavenger receptors and RAGE in astrocytes and microglia were studied by immunocytochemistry (Fig. 4) and immunoblot (Fig. 5). Considering that astrocytes can modulate microglial cells activity and vice versa, the characterization of SRs was performed both on purified cultures of astrocytes and microglial cells and on mixed glial cultures. The immunocytochemistry of mixed glial cultures is shown (Fig. 4), because no differences were found in the expression of any of the SRs tested between glial cells in isolated cultures and in mixed cultures (data not shown). Both microglia (lectin-positive cells in Fig. 4B) and astrocytes (lectin-negative in Fig. 4B) were labeled by an antibody against SR-BI (Fig. 4A). Immunoblot analysis of microglial and astrocytes cultures showed that anti-SR-B antibody recognized two bands at ~85 and ~60 kDa in both cell types (Fig. 5). In mixed glial cultures, astrocytes (GFAP-positive cells, arrowheads in Fig. 4D) were negative for SR-A, whereas microglia were labeled for SR-A/VII (small arrows in Fig. 4C). Immunoblot showed that anti-SR-A antibody recognized two bands at ~70 and ~55 kDa in microglial cell samples (Fig. 5). RAGE also was expressed only by microglial cells (small arrows in Fig. 4E). Immunoblot analysis confirmed that the anti-RAGE antibody labeled two bands at ~53 and ~60 kDa, present only in microglial cell homogenates (Fig. 5).

The presence of SR-MARCO was evaluated by immunocytochemistry of live cells using two nonrelated antibodies, obtaining similar results. The immunocytochemistry and immunoblot presented in Figs. 4–6 correspond to those performed with the rat anti-mouse SR-MARCO (MCA 1848 from Serotec). Microglia (lectin-positive; small arrows in Fig. 4H) and astrocytes (GFAP-positive, arrowheads in Fig. 4J) were positive for SR-MARCO (Fig. 4, G and I). Immunoblotting with both anti-SR-MARCO antibodies recognized a single (or a doublet in some samples) band at ~54–55 kDa (Fig. 5).

Immunoblot of cell lysates confirmed the results of the immunocytochemical studies. Astrocytes expressed SR-B and SR-MARCO, whereas no positive bands were detected for SR-A and RAGE. Under the same conditions, immunoblotting of microglial lysates demonstrated positive bands in correspondence to the specific control tissues for the four SRs tested (Fig. 5).

Imune Neutralization of SR-MARCO Blocks Adhesion of Newborn Rat Astrocytes to fAβ—In order to support our data indicating that SR-MARCO is the receptor mediating Aβ adhesion, astrocytes were preincubated with an antibody against SR-MARCO with a known neutralizing effect (38). Immune neutralization of SR-MARCO with the IgG blocked glial adhesion to Aβ by 8-fold (p < 0.001), similar to the inhibition observed with fucoidan and poly(I) (Fig. 6). In contrast, preincubation with another antibody used as a control, a rat anti-SR-A IgG, reduced glial adhesion only by 18.7 ± 1.6% (p < 0.001 compared with cells preincubated with anti-SR-MARCO IgG).

Competing Ligands Binding SR-MARCO Inhibit Astrocyte Clearance of Aβ in Vitro—We exposed astrocytes to Cy3-labeled Aβ and nAβ in the presence or absence of poly(I), which blocks SR-mediated binding of Aβ, or its negative control counterpart, poly(C). After 5 h of co-incubation, phagocytosis was not observed under any of the culture conditions tested (Fig. 7A). After 24 h, whereas control and poly(C)-treated astrocytes showed a clear uptake of Aβ, poly(I)-treated astrocytes showed a reduced phagocytosis of Cy3-Aβ (Fig. 7A) and nonlabeled Aβ (Fig. 7B). After 48 h, astrocytes treated with poly(I) phagocyted Cy3-Aβ poorly, compared with the cell-associated fluorescence observed under control conditions or in cultures exposed to poly(C) (Fig. 7A). Besides the strong astrocyte-associated fluorescence, especially in perinuclear vesicles, the Aβ coating of the matrix was cleared in the area surrounding the astrocytes only by astrocytes that were not exposed to poly(I) (Fig. 7, A and B).

| Competing ligand | 37 °C | 20 °C | 4 °C |
|-----------------|-------|-------|------|
| Adherent cells (percentage of control) |       |       |      |
| None            | 100 ± 3.5 | 100 ± 5.1 | 100 ± 4.5 |
| Poly(I)         | 13.5 ± 3.7* | 6.70 ± 2.1* | 10.7 ± 2.6a |
| Poly(C)         | 86.2 ± 10.3 | 91.2 ± 4.6 | 105 ± 5.0 |

* a p < 0.001, Mann-Whitney U test.

**ND, not determined.**
Astrocytes Express SR-MARCO in Brain Sections—To verify the presence of SR-MARCO in brain astrocytes, double immuno-histochemistry for SR-MARCO and GFAP was performed in sections of newborn, juvenile, and adult rat brains (Fig. 8). Astrocytes, identified by staining with rabbit anti-GFAP in brains of juvenile (Fig. 8D) and adult (Fig. 8F) rats, stained specifically for SR-MARCO (Fig. 8, C and E, respectively). The cells co-expressing SR-MARCO and GFAP in juvenile and adult rat brains presented the characteristic star-shaped morphology of astrocytes. In contrast, brain sections of newborn rats also showed SR-MARCO staining (Fig. 8A). However SR-MARCO-positive cells had a bi- or unipolar morphology (arrows), and GFAP staining was very pale (Fig. 8B). These observations confirm that astrocytes in rat brains express SR-MARCO.

**Fig. 4.** Labeling of astrocytes and microglia in mixed glial cultures from neonatal rat in culture by the different scavenger receptors. A and B, double immunofluorescence of mixed glial cultures with anti-SR-BI (Rho) (A) and lectin as a microglial cell identity marker (FITC) (B). Astrocytes (arrowheads) and microglia (small arrows) were positive for anti-SR-BI (A). Only microglia (small arrows) were labeled with lectin (B). C and D, mixed glial culture double immunofluorescence with anti-SR-AI/AII (Rho) (C) and GFAP as an astrocyte identity marker (FITC) (D). Only microglia (GFAP negative in D) expressed SR-AI/AII (small arrows in C and D). Astrocytes (arrowheads in C and D) were not labeled by anti-SR-AI/AII (B). E and F, mixed glial culture double immunofluorescence with anti-RAGE (Rho) (E) and anti-vimentin (FITC) (F). Only microglia were labeled with anti-RAGE (small arrows in E and F). Anti-vimentin labeled both astrocytes (arrowheads in E and F) and microglia (small arrows in E and F). G–J, anti-SR-MARCO labeling of mixed glial cultures. Double immunofluorescence with anti-SR-MARCO (Rho in G and I) and lectin as a microglial cell identity marker (FITC) (H) or GFAP as an astrocyte identity marker (FITC) (J). Both astrocytes (arrowheads) and microglia (small arrows) were labeled with anti-SR-MARCO (G and I). Scale bar, 50 μm.
We show that astrocytes from neonatal rats, like microglia, adhered to surfaces coated with both fAβ and nFAβ. Adhesion to fAβ and nFAβ was concentration-dependent for both cell types. Adhesion and uptake of fAβ by macrophages and microglia is well documented (46, 48). It has also been reported that astrocytes from adult but not from neonatal mice bind and degrade fAβ in culture (36). In contrast, adhesion of astrocytes and microglia to nFAβ is not well established. Previous reports have only shown that nonfibrillar oligomeric Aβ (1-42) was able to trigger glial activation, which can lead to the production of potentially toxic molecules, such as inflammatory cytokines (interleukin-1β) and nitric oxide (49). It is interesting that gial cells adhered to nFAβ, because nFAβ can spontaneously form insoluble assemblies of β-pleated sheets (fibrillar) similar to the amyloid fibrils found in AD brains. Binding of nFAβ to glial cells can lead to the internalization of Aβ through a scavenger receptor. nFAβ uptake could have two possible consequences: (i) the degradation of internalized nFAβ, which could decrease the formation of fAβ, or (ii) accumulated nFAβ inside glial cells, which could trigger further aggregation of amyloid fibrils.

Normal subjects and AD patients have similar concentrations of soluble Aβ in the cerebrospinal fluid (50), and Aβ expression by cells in culture is constitutive (51, 52). However, the accumulation of amyloid could depend on the rate of degradation of Aβ, even in the absence of changes of Aβ production. It has been reported that soluble Aβ is internalized by microglia from newborn mice, through receptors that are not blocked by fucoidan and acetyl-LDL (53). It was suggested that microglia internalized soluble Aβ via pinocytosis, in contrast to receptor-mediated endocytosis of fAβ (48). In contrast to that report, we found that microglia and astrocyte adhesion to nFAβ was specifically inhibited by some SR ligands.

Fucoidan and poly(I) similarly inhibited adhesion of both microglia and astrocytes to surfaces coated with fAβ and nFAβ.

In contrast, oxLDL and Mel-BSA inhibited better the adhesion of astrocytes and microglia to nFAβ than to fAβ. The different efficacy as competitors of ligands with a wide range of SR-sensitivity, such as oxLDL and Mel-BSA, suggests that at least some of the receptors involved in the interaction of glial cells with fAβ and nFAβ are different. The involvement of different SRs could result in different glial cell activation patterns. The fact that preincubation of cells with competing ligands at 37, 20, and 4 °C gave similar results argues against the possibility that the decrease in adhesion to Aβ could be due to the endocytosis of receptor complexes, resulting in decreased cell surface receptor expression. Our results indicate that competing ligands are blocking Aβ-SR interaction.

On the other hand, native LDL did not inhibit the adhesion of glial cells to fAβ or nFAβ. This result suggests that the adhesion of neonatal astrocytes to fAβ and nFAβ was mediated by a receptor different from SR-BI (since SR-BI binds native LDL) (54) as previously suggested for astrocytes from adult mice (36). The authors reported that fucoidan and polyinosinic acid inhibited adhesion of astrocytes to Aβ, but neutralizing antibodies blocking SR-BI had no effect on Aβ binding. We used 100 μg/ml LDL to compete for SR-BI binding, 5-fold the concentration needed to attain maximal binding of COS cells transfected with SR-BI (55). In microglia from an SR-A knockout newborn mouse expressing SR-BI (56), anti-SR-BI only partially blocked whereas fucoidan totally blocked adhesion of SR-A−/− microglia to Aβ. Our results also suggest that a fucoidan-sensitive receptor other than SR-A and SR-BI could mediate the adhesion of glia to surfaces coated with Aβ.

Scavenger-like receptors binding Aβ other than SR-BI are present in astrocytes, including low density lipoprotein receptor-related protein (5) and membrane-associated proteoglycans. We propose that SR-MARCO, an SR closely related to SR-AI (33, 57), that we immunodetected in astrocytes and microglia could bind Aβ. This proposition is strongly supported by the fact that a neutralizing antibody for SR-MARCO prevented adhesion of glial cells to Aβ. The magnitude of the adhesion inhibition was similar to that observed with poly(I) and fucoidan. In contrast, anti-SR-AI/II IgG had no effect on adhesion of astrocytes to Aβ. These findings lead us to conclude that SR-MARCO is probably the member of the SR-A family promoting astrocyte adhesion to Aβ.

We found that neonatal astrocytes expressed SR-BI and SR-MARCO but not SR-AI/II or RAGE. In contrast, microglia presented all of them. Previous reports showed that murine neonatal microglia in culture express SR-A, SR-BI, and CD36 (48), whereas adult mouse microglia only express low levels of CD36 (2) and do not express detectable levels of SR-A (58). On the other hand, the expression of receptors also changes under certain pathophysiological conditions. SR-A is expressed by activated microglia in the vicinity of Aβ-containing senile plaques (59, 60). Astrocytes from adult mouse and human brains and from AD brains express SR-BI (15). We presented the first description of the presence of SR-MARCO and SR-BI in astrocytes from neonatal rat in culture. However, the extrapolation of results obtained with microglia or astrocytes from neonatal animals to the adult condition must be done with caution, because the
pattern of SR expression changes with age (2, 36). We also confirmed SR-MARCO expression in the brains of rats from neonatal to adult stages. Astrocytes in the juvenile and adult rat express SR-MARCO. The presence of SR-MARCO in the brain raises new questions relevant to brain cell physiology in health and disease. In brain slices, the astrocytes expressing SR-MARCO were mostly GFAP-positive. This pattern of expression suggests that SR-MARCO was expressed mainly by activated astrocytes. The restricted pattern of expression of SR-MARCO is not contradictory with the expression of SR-MARCO by astrocytes in culture. In fact, there is a deregulation of GFAP expression in astrocytes in culture, and nearly all astrocytes are GFAP-positive. An expression of SR-MARCO restricted to activated astrocytes could have important consequences for the proposition that it may be involved in the interaction of Aβ with glial cells in Alzheimer disease. The more restricted expression of SR-MARCO relative to the other SR-As in normal tissue suggests that its expression by astrocytes in the human brain and on astrocytes surrounding amyloid plaques will be worth examination.

FIG. 7. SR-A-competing ligands inhibit phagocytosis and clearance of Aβ by astrocytes in vitro. Shown is fluorescence microscopy of astrocytes plated on a glass slide coated with Cy3-labeled Aβ (A) or unlabeled nfAβ (B) as described under “Experimental Procedures.” The Aβ removal assay was performed with astrocytes under control conditions or exposed to poly(I) or poly(C). A, double fluorescence microscopy of neonatal rat astrocytes plated on poly-l-lysine plus Cy3-labeled Aβ-coated surfaces for 5, 24, and 48 h. In the merged image, red corresponds to the Cy3-labeled Aβ, and blue corresponds to the Hoechst-stained nucleus. B, neonatal rat astrocytes plated on poly-l-lysine plus unlabeled nfAβ-coated surfaces for 24 h. Cells were double immunolabeled with an antibody recognizing GFAP (as an identity marker for astrocytes, in green) and an antibody against Aβ (in red). Hoechst-stained nuclei are shown in blue. Merged images allow viewing of the uptake of Aβ by the astrocytes, a process greatly reduced in cells exposed to poly(I).
The possible functional role for astrocyte adhesion to Aβ is another issue. Whereas it has been shown that microglia bind to and internalize Aβ and can degrade it under certain conditions, little is known regarding such activity by astrocytes. We found that neonatal rat astrocytes phagocytosed and cleared surface-bound Aβ, phagocytosis that was partially inhibited by poly(I). These observations, the expression of SR-MARCO by GFAP-positive astrocytes, and the presence of Aβ in astrocytes surrounding amyloid plaques (61–64) suggest that astrocytes could participate in the degradation of Aβ in AD or that dysregulation of Aβ clearance by astrocytes may be responsible for its accumulation in AD. A rapid clearance of apoptotic cells, oxidized compound, and other typical SR ligands from the central nervous system is needed to prevent further damage and disease. In the phagocytosis assay, also the astrocytes exposed to poly(I) phagocytosed Aβ after 48 h. However, the phagocytosis was slower, and the astrocytes were unable to clear the surface-bound Aβ as was observed for control and poly(C)-treated astrocytes. The partial Aβ uptake could depend on the up-regulation of other SR. It is necessary to consider that the poly(I) used to block Aβ uptake binds to the SR and can mediate cell activation. Astrocytes could interact with Aβ without necessarily performing phagocytosis. Astrocytes become rapidly activated in response to various injuries. In many diseases, astrocyte activation has been associated with induction of chemokines and inflammatory cytokines.

An aspect that deserves a closer look is the activation of signaling pathways associated with the binding of SR. We speculate that although Aβ binds to several different SRs, differences in cell response could depend on the activation of specific intracellular signaling pathways. Although there is no information regarding SR-MARCO, ligand binding to related SRs like SR-A stimulates activation of phosphatidylinositol 3-kinase and protein-tyrosine kinase and mitogen-activated protein pathways (65, 66), and protein kinase C inhibitors reduced myelin phagocytosis mediated by SR-A and complement receptor (67). Binding to SR-A also stimulates increases in proinflammatory cytokines, such as interleukin-1β and tumour necrosis factor α (65, 66), related to neurotoxicity in degenerative diseases. It has been well established that astrocytes regulate microglial activation by Aβ and proinflammatory molecules, including the production of reactive oxygen species (63, 64, 68), and they also influence the interaction of microglia with Aβ, changing the phagocytic capability of microglial cells (69) and microglial cell-mediated neurotoxicity (70). However, it is still unclear whether reactive astrocytes accumulating at sites of Aβ deposition have neuroprotective or damaging functions (71). Although astrocytes do not produce reactive oxygen species (15, 56), they produce important amounts of nitric oxide when they adhere to matrices containing Aβ. Astrocytes and microglial cells could also influence their expression pattern for different SRs. However, at least under our culture conditions, we did not find differences in the expression of SR-A, SR-B, SR-MARCO, or RAGE between astrocytes and microglia in isolated cultures and those in mixed cultures.

In summary, considering the findings regarding the adhesion pattern of astrocytes and microglia to Aβ and pAβ (poorly examined by previous work), the fact that binding of neonatal rat microglia and astrocytes is mediated via fucoidan- and poly(I)-sensitive mechanisms, and the expression of SR-MARCO in their surface both in culture and in the rat brain, we propose that SR-MARCO could be the scavenger receptor responsible for the uptake of Aβ by astrocytes and microglia. We are presently evaluating if the activation of microglia or astrocytes by proinflammatory cytokines or Aβ induces the up-regulation of SR-MARCO. We are also studying the activation of specific signal transduction pathways in response to Aβ binding.

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