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Signaling Pathways in the Activation of Mast Cells Cocultured with Astrocytes and Colocalization of Both Cells in Experimental Allergic Encephalomyelitis

Dae Yong Kim,* Dool Jeoung,† and Jai Youl Ro*

Mast cells in the CNS participate in the pathophysiology of chronic neurodegenerative inflammatory diseases. This study aimed to investigate the signaling pathway of mast cells activated in an environment cocultured with astrocytes and to explore the role of their colocalization in brain of experimental allergic encephalomyelitis. Human mast cell line-1 cells and human U87 glioblastoma cells (U87) or mouse bone marrow-derived mast cells and mouse cerebral cortices-derived astrocytes were cocultured. Intracellular Ca2+ was measured by confocal microscopy; histamine by fluorometric analyzer; leukotrienes by ELISA; small GTPases, protein kinase Cs, MAPK, c-kit, CD40, and CD40L by Western blot; NF-κB and AP-1 by EMSA; cytokines by RT-PCR; and colocalization of mast cells and astrocytes in brain by immunohistochemistry. Mast cells cocultured with astrocytes showed time-dependent increases in intracellular Ca2+ levels, release of histamine and leukotrienes, and cytokine production. Mast cells or astrocytes showed enhanced surface expression of CD40L and CD40, respectively, during coculture. Mast cells cocultured with astrocytes induced small GTPases (Rac1/2, cdc42), protein kinase Cs, MAPK, NF-κB, and AP-1 activities. These changes were blocked by anti-CD40 Ab pretreatment or CD40 small interfering RNA. Mast cells increased in the thalamus of experimental allergic encephalomyelitis model, particularly colocalized with astrocytes in the thalamic border region of the habenula. In conclusion, the data suggest that activation of mast cells cocultured with astrocytes induces release of mediators by small GTPases/Ca2+ influx through CD40–CD40L interactions to participate in the pathophysiology of chronic neurodegenerative inflammatory diseases, such as multiple sclerosis. The Journal of Immunology, 2010, 185: 273–283.

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Address correspondence and reprint requests to Dr. Jai Youl Ro, Department of Pharmacology and Samsung Biomedical Research Institute, Sungkyunkwan University School of Medicine (to J.Y.R.).

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Abbreviations used in this paper: + anti-CD40, anti-CD40 Ab pretreatment; 2-APB, 2-aminoethoxydiphenyl borate; 8-oxo-dG, 8-hydroxydeoxyguanosine; a, negative control; b, competition assay; BBB, blood-brain barrier; BM, BMMCs cultured alone; BMMC, bone marrow-derived mast cell; [Ca2+]i, intracellular Ca2+; CD40 siRNA, HMC-1 cells cocultured with transfected U87 cells; EAE, experimental allergic encephalomyelitis; GFAP, glial fibrillary acidic protein; HMC, HMC-1 cells cultured alone; HMC-1, human mast cell line-1; I, LT, leukotriene; MIG, monokine induced by IFN-γ; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; PBS, PBS containing 0.1% Tween 20; PKC, protein kinase C; siRNA, small interfering RNA.

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can produce cytokines or chemokines that cause mast cell degranulation (16). We therefore hypothesized that mast cells may interact with astrocytes via interactions of cell surface molecules in the brain. To pursue this hypothesis, we investigated the signaling pathways activated in mast cells during coculture with astrocytes. We demonstrated that mast cells were activated with astrocytes through cross-talk of CD40–CD40L.

Materials and Methods

Cell line culture

Human mast cell line-1 (HMC-1) cells were provided by J. H. Butterfield (Minneapolis, MN). Cells were grown in IMDM (Life Technologies, Carlsbad, CA) containing 10–5 M monothioglycerol, 100 U/ml penicillin/streptomycin (Life Technologies), and 10% heat-inactivated FBS at 37°C in a 5% CO2 atmosphere. These culture conditions were designated as control medium.

U87 glioblastoma cell lines were obtained from Korea Cell Line Bank (Seoul, Korea); grown in DMEM (Life Technologies) formulated with 4.5 mM t-glutamate, 4.5 mg/ml t-glucose, and 110 mg/ml sodium pyruvate; and supplemented with 10% FBS and 100 U/ml penicillin/streptomycin.

Preparation of bone marrow-derived mast cells and primary astrocytes

Bone marrow cells were flushed from femurs and tibia of BALB/c mice (female, 8 wk old). RBCs were lysed using 0.1 M NH4Cl, and the remaining cells were washed, resuspended, and cultured in RPMI 1640 supplemented with 10% FBS and 50% WEHI-3B conditioned media, which contains IL-3, for 5 wk. Bone marrow-derived mast cells (BMMCs; 5 × 106 cells) were collected onto object glasses by cytospin (400 g, 3 min). BMMCs were fixed in methanol for 2–3 min and then stained with May-Grünwald solution for 15 min, followed by Giemsa solution for 10 min and by washing steps in H2O, confirmed under microscope (21).

Primary astrocytes were prepared from the cerebral cortices of 1-d-old BALB/c mice. In brief, animals were sacrificed by decapitation, meninges were removed, and cortices were minced and gently dissociated in HBSS. Cells were supplemented with DMEM containing 5% FBS, plated onto 75-cm2 culture flask (5 × 104 cells/dish), and incubated at 37°C in a humidified atmosphere of 95% air, 5% CO2. After 14 d of culture, floating microglia was removed by shaking the flask vigorously. As reported previously (22), >95% of cells were stained for astrocyte-specific glial fibrillary acidic protein (GFAP, Sigma-Aldrich, St. Louis, MO).

Coculture of mast cells and astrocytes

U97 cells or primary astrocytes (1 × 106 cells) were grown in 75-cm2 flask until confluent, and then HMC-1 cells or BMMCs (3 × 106 cells) were added to each astrocyte flask, and the cells were cocultured for up to 24 h. In vivo, brain astrocytes outnumber mast cells, and we chose a 3:1 ratio of mast cells and astrocytes to activate mast cells and produce sufficient numbers for testing. Supernatants after coculture were collected and kept at −70°C until analysis for mediators (histamine, leukotrienes [LTs], and cytokine array).

Histamine assay

Histamine in supernatants obtained from cocultured cells was quantified using an automated fluorometric analyzer (Astoria analyzer series 300, Clarikan, OR) (23). The detection limit of the histamine assay was ≈5 ng/ml, and the amount of histamine released was expressed as a percentage of the total histamine content in unstimulated mast cells.

LT immunoassay

The LT content of supernatants obtained from cocultured cells was determined using an enzyme immunoassay kit according to the manufacturer’s instructions. Briefly, 50 µl samples were incubated with anti-LT antiserum (diluted 1/120) and acetylcholinesterase-linked LTs (diluted 1/120) in wells that had been coated with mouse mAbs for 18 h at room temperature. After rinsing with washing buffer, color was developed using Ellman’s reagent, and the plates were read at 412 nm with a spectrophotometer. The concentrations of LTs were then calculated using standard curves generated with specific LT standards. The results are expressed in ng/ml (1 × 106 cells). The LT concentration was calculated using analysis tools on the Cayman Chemical (Ann Arbor, MI) Web site (www.caymanchem.com/app/template/analysis%2CEDIA.xml/).

Cytokines secreted in media after coculture using cytokine Ab array

The membranes included in a human protein cytokine array kit (RayBiotech, Norcross, GA) were blocked with a blocking buffer, and then 1 ml cocultured media was added and incubated at room temperature for 2 h. After incubation, each membrane was thoroughly washed with the provided washing buffers and incubated for 1–2 h at room temperature with biotin-conjugated anti-cytokine Ab, followed by the incubation of streptavidin-HRP conjugate. After several washes, cytokine–Ab complexes were detected by chemiluminescence using reagents and procedures provided by RayBiotech. Levels of individual cytokines were assessed semiquantitatively using a LAS-3000 (Fujifilm, Tokyo Japan).

GST effector pull-down assay

Small GTPase protein activities were assayed, as previously described (25), using EZ-Detect protein activation kits (Upstate Biotechnology, Lake Placid, NY). Cocultured HMC-1 cells or BMMCs (3 × 106 cells) were suspended in 0.5 ml lysis buffer (25 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM MgCl2, 1% Nonidet P-40, 1 mM DTT, 5% glyceral, 1 mM PMSF, 1 µg/ml aprotinin, and 1 µg/ml leupeptin) for 30 min on ice, and supernatants were obtained by centrifugation (13,000 × g for 20 min). According to the manufacturer’s protocol, the active form of small GTPase proteins was obtained from the supernatants by affinity precipitation using Pak-1 protein binding domain, which was fused to GST, and visualized by Western blot analysis with anti-rabbit Rac1/2, cdc42 (1/1000).

Western blot

Cocultured HMC-1 cells or BMMCs (3 × 106 cells/50 ml) were homogenized in lysis buffer (10 mM HEPEs [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 2.0 µg/ml aprotinin, and 2.0 µg/ml leupeptin), and allowed to swell on ice for 30 min. Cell lysates (µg) were subjected to 8–10% SDS-PAGE and transferred to nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, United Kingdom). Membranes were washed with PBS containing 0.1% Tween 20 (PBS-T) and then blocked for 1 h in PBS containing 5% skin milk. After washing the membranes with PBST, they were treated with Abs against PKC isoforms, ERK, JNK, p38, c-kits (Santa Cruz Biotechnology, Santa Cruz, CA), CD40, and CD40L (NeoMarkers, Fremont, CA), and p-PKC isoforms, ERK, JNK, and p38, or 8-galactosidase-linked PKC isoforms, ERK, JNK, and p38, or 8-galactosidase-linked PKC isoforms, ERK, JNK, and p38, or 8-galactosidase-linked PKC isoforms, ERK, JNK, and p38, or 8-galactosidase-linked PKC isoforms, ERK, JNK, and p38, or 8-galactosidase-linked PKC isoforms, ERK, JNK, and p38, or 8-galactosidase-linked PKC isoforms, ERK, JNK, and p38, or 8-galactosidase-linked PKC isoforms, ERK, JNK, and p38, or 8-galactosidase-linked PKC isoforms, ERK, JNK, and p38, or 8-galactosidase-linked PKC isoforms, ERK, JNK, and p38, or 8-galactosidase-linked PKC isoforms, ERK, JNK, and p38, or 8-galactosidase-linked PKC isoforms, ERK, JNK, and p38, or 8-galactosidase-linked PKC isoforms, ERK, JNK, and p38, or 8-galactosidase-linked PKC isoforms, ERK, JNK, and p38, or 8-galactosidase-linked PKC isoforms, ERK, JNK, and p38, or 8-galactosidase-linked PKC isoforms, ERK, JNK, and p38, or 8-galactosidase-linked PKC isoforms, ERK, JNK, and p38, or 8-galactosidase-linked PKC isoforms, ERK, JNK, and p38, or 8-galactosidase-linked PKC isoforms, ERK, JNK, and p38, or 8-galactosidase-linked PKC isoforms, ERK, JNK, and p38, or 8-galactosidase-linked PKC isoforms, ERK, JNK, and p38, or 8-galactosidase-linked PKC isoforms, ERK, JNK, and p38, or 8-galactosidase-linked PKC isoforms, ERK, JNK, and p38, or 8-galactosidase-linked PKC isoforms, ERK, JNK, and p38, or 8-galactosidase-linked PKC isoforms, ERK, JNK, and p38, or 8-galactosidase-linked PKC isoforms, ERK, JNK, and p38, or 8-galactosidase-linked PKC isoforms, ERK, JNK, and p38, or 8-galactosidase-linked PKC isoforms, ERK, JNK, and p38, or 8-galactosidase-linked PKC isoforms, ERK, JNK, and p38, or 8-galactosidase-linked PKC isoforms, ERK, JNK, and p38, or 8-galactosidase-linked PKC isoform...
isofoms, p-ERK, p-JNK, and p-p38 (Cell Signaling Technology, Beverly, MA) diluted with PBST (1:1,000), and incubated for 60 min at room temperature. Membranes were washed with PBST, and treated with HRP-conjugated goat anti-mouse or HRP-conjugated rabbit anti-goat IgG (diluted to 1/5,000 to 1/10,000; Zymed Laboratory, San Francisco, CA) in PBST for 60 min. After washing, the protein bands were visualized using electrogenerated chemiluminescent (ECL) solution (Amerham Biosciences) (25).

Cytokine levels secreted in media after coculture
Amounts of cytokines and chemokines (IL-1β, IL-4, IL-6, TNF-α, IFN-γ, MCP-1, monokine induced by IFN-γ [MIG], and RANTES) secreted into coculture media were determined using an ELISA kit. Coculture media were centrifuged at 400 g for 5 min, and then supernatants were collected. Standard curves were generated using different concentrations of the recombinant cytokines. The limit of detection of this method was >7.8 pg/ml.

RT-PCR
Total cellular RNA was isolated from cocultured HMC-1 cells or BMMCs (3 × 10^6 cells) using TRI-Reagent (Molecular Research Center, Cincinnati, OH). RT-PCR was performed in a final volume of 50 μl using amphi
tvert one-step RT-PCR kit (GenDEPOT, Barker, TX) in an automated thermal cycler (BIOER Xp, BIOER Technology, Hangzhou, China). PCR assays were performed for 35 cycles. Each cycle consisted of the following steps: denaturation at 94°C for 30 s, annealing at 56°C for 45 s, and extension at 72°C for 1 min. PCR products were analyzed using 1% agarose gel containing ethidium bromide (25).

The primer sequences used were as follows: human IL-6 sense, 5′-GCC TTT GGA GCA TTT TGT-3′ and antisense, 5′-GCA TGA GAT GAG TGG TTG TC-3′; mouse IL-6 sense, 5′-TGG AGC CAT AGC AGT GAG TTC GGT CCA GTT C-3′ and antisense, 5′-TTA TTC TCT AGC TCC ACG CC-3′; mouse TNF-α sense, 5′-TTC GGA CTA GGA AAA CGA TGC-3′ and antisense, 5′-TGG GTT TGG TGC TAC GGC GTT-3′; mouse MCP-1 sense, 5′-GCC GGC ACC TGT CCA GCC TCA-3′ and antisense, 5′-GGT TGC TGT GGA GCC ACC AT-3′; human MIG sense, 5′-ATG TCC ACC CAA CCA CAT CC-3′ and antisense, 5′-CAC TGT GGA GGA AAC AGG GA-3′; mouse MIG sense, 5′-AGA CAT CAG TCC TGC CAT GAA GTC-3′ and antisense, 5′-GGT GCC AGC GAT TGG TTA-3′; human GAPDH sense, 5′-GGA GGA ATG GGT CCT GCA GAT-3′ and antisense, 5′-AGC GGT CAA CTT CAC ATT CA-3′; human GAPDH sense, 5′-ATG TCC ACC CAA CCA CAT CC-3′ and antisense, 5′-CAC TGT GGA GGA AAC AGG GA-3′; mouse MIG sense, 5′-AGA CAT CAG TCC TGC CAT GAA GTC-3′ and antisense, 5′-GGT GCC AGC GAT TGG TTA-3′; human MIG sense, 5′-ATG TCC ACC CAA CCA CAT CC-3′ and antisense, 5′-CAC TGT GGA GGA AAC AGG GA-3′; mouse MIG sense, 5′-AGA CAT CAG TCC TGC CAT GAA GTC-3′ and antisense, 5′-GGT GCC AGC GAT TGG TTA-3′; human GAPDH sense, 5′-GGA GGA ATG GGT CCT GCA GAT-3′ and antisense, 5′-AGC GGT CAA CTT CAC ATT CA-3′; mouse GAPDH sense, 5′-AAC TGT GGC ATT GTG GAA GG-3′ and antisense, 5′-ACA CAT TGG GGG TAG GAA CA-3′.

EMSA
Nuclear extracts were prepared from cocultured HMC-1 cells or BMMCs (3 × 10^6 cells). Cells were washed twice with ice-cold PBS, and resuspended in 1 ml ice-cold buffer A (10 mM HEPES/KOH [pH 7.9], 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM DTT, 0.2 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml aprotinin). After incubation on ice for 15 min, the cells were lysed by adding Nonidet P-40 (10% v/v) Nonidet P-40, to a final concentration of 0.625%, v/v) and immediately vortexed for 10 s. Nuclei were harvested by centrifugation at 20,000 g for 10 min. Nuclei were resuspended in 1 ml ice-cold buffer B (20 mM HEPES/KOH [pH 7.9], 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 25% glycerol, 0.2 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml aprotinin). After incubation at 4°C for 20 min on a shaking platform, the nuclei were clarified by centrifugation at 15,000 × g for 10 min. The supernatant (nuclear extract) was then transferred to a new tube, and quantified using Bradford’s method. Nuclear extracts were mounted using aqueous mounting medium, and examined using a confocal microscope (LSM 5 EXCITeR; Carl Zeiss) (28).

Statistical analysis
Experimental data are shown as means ± SEM. An unpaired Student’s t test was used to analyze the results for statistical significance when only two conditions were compared. Values of p below 0.05, 0.01, or 0.001 were considered significant.

Results
[Ca²⁺], level in mast cells cocultured with astrocytes
Increased [Ca²⁺], levels are necessary for the degranulation of preformed mediators and the secretion of newly synthesized mediators in activated mast cells. Coculture with U87 cells or primary astrocytes increased [Ca²⁺], level in HMC-1 cells or BMMCs, respectively, as assessed by confocal microscopy (Fig. 1A). The cocultured HMC-1 cells (Fig. 1B) or BMMCs (Fig. 1C) increased [Ca²⁺], levels in a time-dependent manner. The [Ca²⁺], levels in both mast cells reached plateau at 60 min (from 3.1 ± 0.35 to 16.6 ± 0.65 × 10³ 3 OD in cocultured HMC-1 cells, and from 5.12 ± 0.12 to 10³ to 26.7 ± 0.96 × 10³ OD in cocultured BMMCs).

Effects of anti-CD40 Ab on [Ca²⁺], levels in mast cells cocultured with astrocytes
CD40 and CD40L are expressed on diverse cell types from hematoopoietic and nonhematopoietic compartments to regulate immune
FIGURE 1. Activation of mast cells cocultured with astrocytes. HMC-1 cells or BMMCs (3 × 10^6 cells) were cocultured with U87 cells or primary brain astrocytes (1 × 10^6 cells), respectively, for the indicated periods. The anti-CD40 Ab (300 ng/ml) was pretreated in astrocytes 1 h before coculture, and CD40 siRNA was performed, as described in Materials and Methods. Fluo-3-acetoxymethyl ester (5 μM) was added to cocultured HMC-1 cells or BMMCs and incubated for 30 min. The [Ca^{2+}]_i level in cocultured HMC-1 cells or in BMMCs was analyzed by confocal microscopy. Histamine and LTs released from both cocultured mast cells were determined using an automated fluorometric analysis and ELISA, respectively. CD40 and CD40L molecules were determined by Western blot. A, Intensity of fluorescence in confocal microscopy. B and C, Time course of [Ca^{2+}]_i in cocultured HMC-1 cells or BMMCs. D, Expression of CD40 or CD40L in cocultured HMC-1 cells and U87 cells. E and F, Histamine and LT release. G and H, Histamine and LT release after anti-CD40 Ab pretreatment. The data are representative of four independent experiments (n = 4). *p < 0.01; **p < 0.001 versus HMC-1 cells or BMMC culture alone; *p < 0.01; **p < 0.001 versus cocultured HMC-1 cells or cocultured BMMCs or anti-CD40 Ab pretreatment; ##p < 0.01; ###p < 0.001 versus cocultured HMC-1 cells. ♦ Numbers below bands are the ratio of band density of each group (CD40 or CD40L) versus those of control and tubulin protein. + anti-CD40, anti-CD40 Ab pretreatment; BMMCs, BMMC culture alone; CD40 siRNA, HMC-1 cells cocultured with transfected U87 cells; coculture; HMC-1 cells or BMMCs cocultured with each astrocyte; HMC-1, HMC-1 cell culture alone.
responses. Mast cells express CD40L, a potent inducer of astrocyte activation (29), and astrocytes functionally express CD40. Cocultures of HMC-1 cells showed higher levels of CD40L and similar levels of CD40, as assessed via Western blot. Coculture increased CD40 expression in astrocytes (U-87 cells), but CD40L was not detected (Fig. 1D).

Therefore, we examined whether 300 ng/ml anti-CD40 Ab pretreatment for 1 h (optimal dose and time were 300 ng/ml and 1 h, respectively) (Supplemental Fig. 1A, 1B) inhibited [Ca^{2+}]_i levels in cocultured HMC-1 cells. Anti-CD40 Ab pretreatment time dependently decreased [Ca^{2+}]_i, levels in cocultured HMC-1 cells (Fig. 1B) or BMMCs (Fig. 1C), but did not completely inhibit [Ca^{2+}]_i levels in activated mast cells. Similarly, CD40 siRNA (Supplemental Fig. 7) decreased [Ca^{2+}]_i levels in cocultured HMC-1 cells (Fig. 1B), whereas the IgG Ab control did not affect [Ca^{2+}]_i levels in HMC-1 cells or BMMCs (Supplemental Fig. 1C, 1D).

**Effects of anti-CD40 Ab on mediator releases in mast cells cocultured with astrocytes**

Ca^{2+} influx is essential for releasing proinflammatory mediators, such as histamine and LTs, during mast cell activation (23) and inducing some inflammatory cytokine production (30). Therefore, we examined the release of histamine and LTs in mast cells cocultured during the period of time indicated. Histamine release (in ng/10^4 cells) in cocultured HMC-1 cells was 51.3 ± 7.32 at 1 h, 93.4 ± 8.14 at 3 h, 161.2 ± 10.318 at 5 h, and 210.79 ± 16.32 at 12 h compared with basal levels of 23.0 ± 0.22. In cocultured BMMCs, histamine release (in ng/10^4 cells) was 109.5 ± 9.20 at 1 h, 209.5 ± 20.20 at 3 h, 271.3 ± 16.12 at 5 h, and 280.9 ± 11.19 at 12 h compared with controls (23.1 ± 0.25) (Fig. 1E).

LTs (in pg/10^4 cells) secreted in cocultured HMC-1 cells were 28.6 ± 4.10 at 5 h, 48.5 ± 2.50 at 12 h, and 47.4 ± 3.15 at 24 h compared with control (2.8 ± 0.30). In cocultured BMMCs, amounts of LTs were 45.4 ± 1.30 at 5 h, 51.8 ± 1.65 at 12 h, and 56.8 ± 1.35 at 24 h compared with control (5.6 ± 0.90) (Fig. 1F).

Anti-CD40 Ab pretreatment decreased the histamine release (in ng/10^4 cells) by 43–54% (25.2 ± 1.65 at 1 h; 42.7 ± 6.30 at 3 h; 87.8 ± 7.29 at 5 h; and 115.1 ± 8.52 at 12 h), compared with cocultured HMC-1 cells (74.3 ± 4.18 at 1 h; 178.7 ± 5.63 at 3 h; 192.1 ± 12.32 at 5 h; and 208.3 ± 13.12 at 12 h). In cocultured BMMCs, anti-CD40 Ab pretreatment also decreased the histamine release (in ng/10^4 cells) by 54–70% (40 ± 9.60 at 1 h; 60 ± 14.60 at 3 h; 90.4 ± 17.26 at 5 h; and 134.7 ± 13.03 at 12 h), compared with cocultured BMMCs (99.5 ± 12.20 at 1 h; 199.5 ± 8.20 at 3 h; 261.3 ± 15.28 at 5 h; and 293.9 ± 18.93 at 12 h) (Fig. 1G). Anti-CD40 Ab showed similar inhibition of LT secretion (Fig. 1H).

**Effects of anti-CD40 Ab on the expressions of cytokines and chemokines in the mast cells cocultured with astrocytes**

We next measured cytokine and chemokine levels secreted in the media of HMC-1 and U87 cells cocultured for 12 h, as assessed by cytokine array kit (Supplemental Fig. 2). Coculture increased IL-6, TNF-α, MCP-1, MIG, and RANTES levels by 2.6-, 25.9-, 7.0-, 2.2-, and 3.4-fold, respectively. mRNA expression of IL-6, TNF-α, MCP-1, MIG, and RANTES peaked at 12 h in cocultured HMC-1 cells (Fig. 2A, left panel) and 6 h in BMMCs (Fig. 2A, right panel), as assessed by RT-PCR, as did protein levels (Supplemental Fig. 3). Anti-CD40 Ab pretreatment blocked this increase in cytokine mRNA levels in the cocultured HMC-1 cells (Fig. 2B, left panel) and BMMCs (Fig. 2B, right panel), as well as protein levels in HMC-1 cells (Supplemental Fig. 3).

**Effects of anti-CD40 Ab or CD40 siRNA on the activities of Rho family GTPases in the mast cells cocultured with astrocytes**

Rho family GTPases activate intracellular kinase cascades to modulate gene transcription (31). Coculture time dependently increased the activities of Rho family GTPases (Rac1/2, cdc42) in both mast cells (Supplemental Fig. 4A). Rac1, Rac2, and cdc42 activities peaked at 15, 15, and 30 min in cocultured HMC-1 cells (left panel) or BMMCs (right panel). Anti-CD40 Ab pretreatment blocked the increase in Rac1/2 and cdc42 activity in cocultured HMC-1 cells (Fig. 3A, left panel) and BMMCs (Fig. 3A, middle panel).
The Ca²⁺/PKC pathways activate MAPKs to influence biological responses, including immune responses and proinflammatory cytokine expression (32). Coculture increased phosphorylation of ERK, JNK, and p38, peaking at 60 min in both cell types (Supplemental Fig. 4C). Anti-CD40 Ab inhibited activation of all three MAPKs in cocultured HMC-1 cells (Fig. 3C, left panel) and BMMCs (Fig. 3C, middle panel). CD40 siRNA also inhibited the activities of MAPKs in cocultured HMC-1 cells (Fig. 3C, right panel).

Effects of anti-CD40 Ab on the transcriptional factor activity in the mast cells cocultured with astrocytes

We next measured activation of the transcription factors, NF-κB and AP-1. Coculture increased NF-κB and AP-1 DNA binding in nuclear extracts. NF-κB DNA-binding activity peaked at 120 min, and AP-1 DNA-binding activity peaked at 90 min (Supplemental Fig. 4D). Anti-CD40 inhibited NF-κB and AP-1 activation in cocultured HMC-1 cells (Fig. 3D, left panel) and BMMCs (Fig. 3D, right panel).

Effects of a Rac inhibitor or Ca²⁺ influx inhibitor on Rho family and signaling molecules in the HMC-1 cells cocultured with astrocytes

The 8-oxo-dG is a Rac1/2 and cdc42 inhibitor (33–35). Rac1 increases Ca²⁺ influx in epithelial cells (36). The 8-oxo-dG pretreatment inhibited Rho activation (Fig. 4A) and [Ca²⁺]i levels (Fig. 4B), as well as PKC isoforms and MAPK downstream of PKC and MAPK, we used various inhibitors. PKC inhibitors (5 nM staurosporine and Gö6976) inhibited the phosphorylation of MAPK (Supplemental Fig. 5A, right panel) and PKC isoforms (Supplemental Fig. 5A, left panel). Although MAPK inhibitors (5 μM PD98059, 10 μM SP600125, 10 μM SB203580) inhibited MAPK activation (Supplemental Fig. 5B, right panel), they did not inhibit the phosphorylation of PKC isoforms (Supplemental Fig. 5B, left panel). These results suggest that MAPKs are downstream of PKC isoforms.

To clarify cascades of PKC and MAPK, we used various inhibitors. PKC inhibitors (5 nM staurosporine and Gö6976) inhibited the phosphorylation of MAPK (Supplemental Fig. 5A, right panel) and PKC isoforms (Supplemental Fig. 5A, left panel). Although MAPK inhibitors (5 μM PD98059, 10 μM SP600125, 10 μM SB203580) inhibited MAPK activation (Supplemental Fig. 5B, right panel), they did not inhibit the phosphorylation of PKC isoforms (Supplemental Fig. 5B, left panel). These results suggest that MAPKs are downstream of PKC isoforms.

Effects of anti-CD40 Ab on the activities of MAPKs in the mast cells cocultured with astrocytes

PKCo, βI, and βII activate JNK, MEKK2, and ERK5 (32). Therefore, we examined whether PKC isoforms (α, βI, βII, ζ, and θ/λ) are activated in both mast cells cocultured with both astrocytes. Coculture of HMC-1 cells or BMMCs increased phosphorylation of PKCo, βI, and βII, peaking at 30 min (Supplemental Fig. 4B), but did not change PKCζ and θ/λ phosphorylation (data not shown). Anti-CD40 Ab blocked this increase in PKCζ and βII phosphorylation in HMC-1 cells (Fig. 3B, left panel) and BMMCs (Fig. 3B, middle panel). CD40 siRNA also inhibited PKC activation in cocultured HMC-1 cells (Fig. 3B, right panel).

Effects of anti-CD40 Ab or CD40 siRNA on the activities of PKC isoforms in the mast cells cocultured with astrocytes

CD40 siRNA also inhibited the activities of Rac1/2 and cdc42 in cocultured HMC-1 cells (Fig. 3A, right panel).

Effects of anti-CD40 Ab or CD40 siRNA on PKC isoforms in the mast cells cocultured with astrocytes

PKCo, βI, and βII activate JNK, MEKK2, and ERK5 (32). Therefore, we examined whether PKC isoforms (α, βI, βII, ζ, and θ/λ) are activated in both mast cells cocultured with both astrocytes. Coculture of HMC-1 cells or BMMCs increased phosphorylation of PKCo, βI, and βII, peaking at 30 min (Supplemental Fig. 4B), but did not change PKCζ and θ/λ phosphorylation (data not shown). Anti-CD40 Ab blocked this increase in PKCζ and βII phosphorylation in HMC-1 cells (Fig. 3B, left panel) and BMMCs (Fig. 3B, middle panel). CD40 siRNA also inhibited PKC activation in cocultured HMC-1 cells (Fig. 3B, right panel).

Effects of anti-CD40 Ab or CD40 siRNA on the activities of MAPKs in the mast cells cocultured with astrocytes

The Ca²⁺/PKC pathways activate MAPKs to influence biological responses, including immune responses and proinflammatory cytokine expression (32). Coculture increased phosphorylation of ERK, JNK, and p38, peaking at 60 min in both cell types (Supplemental Fig. 4C). Anti-CD40 Ab inhibited activation of all three MAPKs in cocultured HMC-1 cells (Fig. 3C, left panel) and BMMCs (Fig. 3C, middle panel). CD40 siRNA also inhibited the activities of MAPKs in cocultured HMC-1 cells (Fig. 3C, right panel).

Effects of anti-CD40 Ab or CD40 siRNA on the activation of mast cells cocultured with astrocytes. HMC-1 cells (3 x 10⁶ cells) and U87 cells (1 x 10⁶ cells) were cocultured for 30 or 60 min. Anti-CD40 Ab (300 ng/ml), 8-oxo-dG (300 μg/ml), or 2-APB (100 μM) was pretreated 1 h, 30 min, and 15 min, respectively, before coculture. CD40 siRNA was performed, as described in Materials and Methods. A, The activities of Rho family GTPases by each inhibitor. B, The [Ca²⁺]i level by each inhibitor. *Numbers below each band are the ratio of band density of each group versus those of control and total protein. ***p < 0.001 versus HMC-1 cell culture alone; **p < 0.001 versus cocultured HMC-1 cells. The data are representative of four independent experiments (n = 4).

Effects of anti-CD40 Ab on the phosphorylation of MAPKs in cocultured HMC-1 cells (Fig. 3A, right panel).

Effects of anti-CD40 Ab on the activities of PKC isoforms (Supplemental Fig. 5A, left panel) and PKC isoforms (Supplemental Fig. 5B, left panel). Although MAPK inhibitors (5 μM PD98059, 10 μM SP600125, 10 μM SB203580) inhibited MAPK activation (Supplemental Fig. 5B, right panel), they did not inhibit the phosphorylation of PKC isoforms (Supplemental Fig. 5B, left panel). These results suggest that MAPKs are downstream of PKC isoforms.
Ca$^{2+}$ (Supplemental Fig. 6A, 6B). However, the Ca$^{2+}$ influx inhibitor, 2-aminoethoxydiphenyl borate (2-APB) and inositol 1,4,5-triphosphate receptor antagonist, inhibited Ca$^{2+}$ levels (Fig. 4B), but did not inhibit Rac1/2 and cdc42 activity (Fig. 4A). The 8-oxo-dG mimicked the effect of anti-CD40 Ab pretreatment and CD40 siRNA.

Localization of mast cells in EAE mouse brain

Mast cells are particularly abundant in the meninges, a common site of early inflammatory lesions in EAE (37). We therefore investigated the population of mast cells in EAE lesions (EAE score = 3.1 ± 0.10) using May-Grünwald-Giemsa (Fig. 5A). Mast cells in the EAE mouse brain increased 5-fold over controls.

Similarly, EAE increased the expression of the mast cell markers, c-kit and CD40L, as well as astrocyte markers, GFAP and CD40, in the thalamus (Fig. 5B, 5C). Double labeling with c-kit for mast cells (green) and GFAP for astrocytes (red) showed that mast cells colocalized with astrocytes in the thalamus (Fig. 5D).

Furthermore, we indicated schematic diagrams showing signaling pathways in the activation of mast cells cocultured with astrocytes (Fig. 6).
in vitro using both cell lines and primary cell cultures. Therefore, we hypothesized that interaction between mast cells and astrocytes plays an important role in CNS inflammatory diseases, such as MS, and then we cocultured mast cells and astrocytes, and astrocytes support mast cell viability in vitro (19). The interaction in the surface costimulatory molecules of adjacent two cells, and this activation induces histamine and LT release, expression of cytokines and chemokines by Ca\(^{2+}\)-dependent PKC isoforms, and MAPKs, and NF-κB/AP-1 activities indicate blocking action by anti-CD40 Ab or 8-oxo-dG. The dotted arrows indicate a response not determined in this study.

**Discussion**

This study demonstrated that coculture of mast cells and astrocytes releases mediators through the CD40–CD40L interaction. The CD40–CD40L interaction activated Rho family small GTPases (Fig. 3A) and increased [Ca\(^{2+}\)] levels (Fig. 1A, 1B) to release mediators, such as histamine and LTs, and led to the production of cytokines and chemokines (IL-6, TNF-α, MCP-1, MIG, and RANTES) (Fig. 1E–H) through the activation of PKC, MAPKs, and NF-κB/AP-1 pathways. Our data suggest that interaction between mast cells and astrocytes contributes to the development of neurodegenerative diseases, such as MS, because mast cells and astrocytes colocalized in the thalamus in the EAE model (Fig. 5), although only proximity of two cells is not sole mechanism in demyelination of EAE.

The distribution of mast cells within MS plaques increases levels of mast cell-specific mediators in the cerebrospinal fluid of MS patients, and mast cell populations are increased in demyelinated lesions (38). An increase of mast cell degranulation in MS (39) indicates that mast cells may play an important role in the pathogenesis of MS. Mast cell deficiency significantly reduces the severity of MOG-induced EAE, and the restoration of mast cells are degranulated in response to myelin basic protein and substance P, leading to in vitro demyelination (48, 49). Therefore, it has been suggested that mast cells directly participate in the destruction of the myelin sheath in MS. Furthermore, the cytokines and chemokines expressed by mast cells may affect immune cell trafficking through direct chemotaxis and/or influence adhesion molecule expression on the endothelium. The ability of mast cells to migrate to secondary lymphoid organs also raises the possibility that they can regulate the induction and/or amplification of a polarized Th response (50). It has been reported also that murine mast cell- and TNF-dependent T cell activation can contribute to the progression and intensity of many different immune diseases, such as MS (51), and that human mast cells stimulate the activated T cells that are in contact with them at the BBB (52). These reports support our results that mast cells activated with coculture induced preformed mediators (histamine) (Fig. 1E), mediator synthesis (LT) (Fig. 1F), and cytokines and chemokines (IL-6, TNF-α, MCP-1, MIG, and RANTES) (Fig. 2A, Supplemental Fig. 3), although interaction between mast cells and T cells was not observed in this experiment. Therefore, it can be inferred that the mediators secreted by mast cells activated by astrocytes may alter BBB permeability and induce demyelination in MS.

It also has been reported that one of the most cell-dependent mechanisms of T cell activation requires interaction between costimulatory molecules OX40L and OX40 on mast cells and T cells, respectively, and mast cells and T cells can occur in close proximity during immune responses (51). Furthermore, they reported that CD40L and CD40 molecules were not expressed in BMMCs, but other (53) and our laboratories showed that CD40L and CD40 molecules were expressed in BMMCs (data shown in answer sheet). Moreover, the interaction between CD40 and CD40L plays an important role in signal transduction pathways in humoral and cell-mediated immune responses. Mast cells may support tumor cell expansion through constitutive CD40–CD40L signaling in Waldenström’s macroglobulinemia (29). Blocking CD40–CD40L interactions by anti-CD40L Ab prevents murine or common marmoset EAE disease activity (18, 54). The CD40L (CD154) molecule, another member of the TNF family, is expressed in nasal mast cells in perennial allergic rhinitis (55). CD40L is a potent inducer of astrocyte activation (56). We showed that cocultures of mast cells and astrocytes via CD40–CD40L increased [Ca\(^{2+}\)], levels (Fig. 1B, 1C) and mediator release (Figs. 1G, 1H, 2B). This Ca\(^{2+}\) mobilization and exocytotic release of inflammatory mediators (57) were decreased by anti-CD40 Ab pretreatment, suggesting that mast cells can be directly activated by the CD40–CD40L interaction in two adjacent cells.

Activation of small GTPase signal pathway accounts for some effects of CD40R stimulation (58). We found that small GTPases were activated upstream of Ca\(^{2+}\) influx (Fig. 4A, 4B) in mast cells activated through the CD40–CD40L interaction (Supplemental Figs. 3A, 4A). Rac1 activation increases Ca\(^{2+}\) influx in epithelial cells (36), and mast cells activate PKCs, MAPKs, and NF-κB/AP-1 signal pathways (28). PKC regulates NF-κB–dependent transcription (59).

**FIGURE 6.** Schematic diagrams showing signaling pathways in the activation of mast cells cocultured with astrocytes. The data suggest that mast cells cocultured with astrocytes are activated through CD40–CD40L interaction in the surface costimulatory molecules of adjacent two cells, and this activation induces histamine and LT release, expression of cytokines and chemokines by Ca\(^{2+}\)-dependent PKC isoforms, and MAPKs, and NF-κB/AP-1 activities indicate blocking action by anti-CD40 Ab or 8-oxo-dG. The dotted arrows indicate a response not determined in this study.
We found that coculture induced Ca\(^{2+}\) mobilization (Fig. 1B, 1C) and PKC (\(\alpha, \beta, \beta\II\)) activation (Fig. 3B), which are Ca\(^{2+}\)-dependent PKCs, and increased MAPK activity (Fig. 3C). Finally, coculture activated transcription factors (NF-kB, AP-1) (Fig. 3D) and produced cytokines and chemokines (Fig. 2A, Supplemental Fig. 3). Therefore, our data suggest that mast cells activated through coculture with astrocytes induce histamine and LT release via regulating Rho\([Ca^{2+}]i\)/PKCs/MAPKs to induce production of cytokines and chemokines via NF-kB/AP-1 activities.

Anti-CD40 Ab pretreatment and CD40 siRNA blocked mediator release and signaling (small GTPases, \([Ca^{2+}]i\) level, PKC isoforms, MAPKs). However, the anti-CD40 Ab or CD40 siRNA may not be able to completely block the interaction because anti-CD40 Ab may be degraded or other molecules may interact.

The IgG Ab as a control for the anti-CD40 Ab did not affect cocultures of mast cells (Supplemental Fig. 1C, 1D), eliminating a role for nonspecific Ab binding.

The absence of mast cells in the CNS does not completely prevent EAE (8, 13), in part because mast cells outside the CNS may influence the generation of the anti-MOG T cell response and direct T cell migration to target sites. That is, mast cells may disrupt the BBB by secreting chemoattractants that increase invasion of the T cells into CNS. However, in the rat EAE model, mast cells are increased 3-fold in the brain and significantly more are degranulated (60). We also observed that EAE increased the population of mast cells in the thalamus (Fig. 5A) as well as c-kit expression, a marker of mature mast cells. We also showed extensive expression of surface markers of mast cells and astrocytes (Fig. 5B, 5C) and their colocalization (Fig. 5D), although brain mast cells under physiological conditions have low FceRI and lack c-kit expression, unlike normal mast cells (61, 62). Our data suggest that mast cells and astrocytes in the thalamus may directly interact in close proximity during elevation of mast cell population. However, further work is needed to study these interactions.

In conclusion, mast cells are activated via interaction of CD40–CD40L in the surface of two adjacent cells, and the activated mast cells induce mediator release, such as histamine and LT, and cytokine production, via the Rho family and Ca\(^{2+}\)/PKC isoforms/ MAPKs/NF-\(\kappa\)B or AP-1 signal pathways (Fig. 6). That is, mast cells and astrocytes can occur in close proximity in brain. Mediators released in mast cells activated by astrocytes may contribute to the initiation of BBB destruction and demyelination in MS disease through constitutive/inducible CD40–CD40L interactions, providing a novel therapeutic target for neuroinflammatory diseases.

Disclosures
The authors have no financial interests of interest.

References
SUPPLEMENTAL FIGURE LEGENDS

Supplemental figure 1. Effects of anti-CD40 Ab pretreatment on mast cells co-cultured with astrocytes. HMC-1 cells or BMMCs \(3 \times 10^6\) cells) were co-cultured with U87 cells and primary brain astrocytes \(1 \times 10^6\) cells), respectively, for indicated periods. Anti-CD40 Ab was pretreated with dose of 10, 30, 100 or 300 ng/ml for time periods indicated. Control IgG Ab (300 ng/ml) was pretreated 1 h before co-culture. The \([Ca^{2+}]_i\) level in the co-cultured HMC-1 cells or BMMCs was measured by confocal microscopy. The optimal dose and time of anti-CD40 antibody were 300 \(\mu\)g/ml and 1 h. A, \([Ca^{2+}]_i\) intensity for each dose of anti-CD40 Ab pretreatment. B, Time course after anti-CD40 Ab pretreatment. C, D, \([Ca^{2+}]_i\) level in co-cultured HMC-1 cells or BMMCs by anti-CD40 Ab or IgG Ab pretreatment, respectively. Con, control; control IgG, control IgG Ab pretreatment; anti-CD40, anti-CD40 Ab pretreatment. ***, \(P < 0.001\) versus control; ++, \(P < 0.01\); +++, \(P < 0.001\) versus co-cultured HMC-1 cells. **, \(P < 0.01\); ***, \(P < 0.001\) versus co-cultured HMC-1 cells or co-cultured BMMCs. The data are representative of four independent experiments (n = 4).

Supplemental figure 2. Quantification of cytokine production secreted in media co-cultured with HMC-1 cells and U87 cells. HMC-1 cells \(3 \times 10^6\) cells) and U87 cells \(1 \times 10^6\) cells) were co-cultured for 12 h. Cytokine protein expression secreted into co-cultured media was determined using cytokine array kit. HMC-1, media cultured with HMC-1 cell alone; U87, media cultured U87 cell alone; Co-culture, media co-cultured with HMC-1 cells and U87 cells. Quantification of IL-6, TNF-\(\alpha\), MCP-1, MIG and RANTES secreted into media were 2.6, 25.9, 7.0, 2.2 and 3.4 fold, compared to each cell culture alone. The data are representative of four independent experiments (n = 4). MIG, monokine induced by gamma interferon.
Supplemental figure 3. Effects of the anti-CD40 Ab on cytokines or chemokines release from HMC-1 cells co-cultured with U87 cells. HMC-1 cells (3 × 10^6 cells/ml) were co-cultured with U87 cells (1 × 10^6 cells/ml) for 12 h. Supernatants were harvested for measuring cytokines or chemokines using ELISA kit. Anti-CD40 Ab (300 ng/ml) was pretreated 1 h before co-culture. ***, P < 0.001 versus HMC-1 cells alone or U87 cells alone. ++, P < 0.01; +++, P < 0.001 versus co-cultured media. HMC-1 or U87, HMC-1 and U87 culture alone; Culture, co-culture; αCD40, anti-CD40 pretreatment.

Supplemental figure 4. Time courses for activations of signal molecules in mast cells co-cultured with astrocytes. HMC-1 cells or BMMCs (3 × 10^6 cells) were co-cultured with U87 cells and primary brain astrocytes (1 × 10^6 cells), respectively, for indicated periods. Activities of Rho families, PKC isoforms and MAPKs were subjected in protein extracts from co-cultured mast cell lysates using western blot, and NF-κB and AP-1 activities were determined in nuclear extracts using EMSA as described in "Material and Methods". Left and right panels, time course for HMC-1 cells and BMMCs, respectively. A, Activities of Rho family GTPases (Rac1/2, cdc42). B, Activities of PKC isoforms. C, Phosphorylations of MAPKs. D, NF-κB and AP-1 activities. HMC, HMC-1 cell culture alone; BM, BMMCs culture alone; a, negative control; b, competition assay. *, Numbers below bands are the ratio of each band density of Rac1/2 and cdc42, PKCs, MAPKs, NF-κB or AP-1 versus those of control and total proteins. The data are representative of four independent experiments (n = 4).

Supplemental figure 5. Effects of inhibitors on activities of PKC isoforms or MAP Ks in HMC-1 cells co-cultured with U87 cells. HMC-1 cells (3 × 10^6 cells) and U87 cells (1 × 10^6
cells) were co-cultured for 30 or 60 min. PKC inhibitors (5 nM staurosporine and Gö6976) or MAP kinase inhibitors (50 μM PD98059 for ERK, 10 μM SP600125 for JNK and 10μM SB203580 for p38) were pretreated 10 min before co-culture. Left and right panel, phosphorylation of PKC isoforms and MAPKs, respectively. A, Activities of PKC isoforms and MAPKs by PKC inhibitors. B, Activities of PKC isoforms and MAPKs by MAPKs inhibitors. HMC-1, HMC-1 cell culture alone; co-culture, co-cultured HMC-1 cells. *, Numbers below bands are the ratio of each band density of PKCs or MAPKs versus those of control and total proteins. The data are representative of four independent experiments (n = 4).

Supplemental figure 6. Effects of Rac inhibitor (8-oxo-dG) on the activation of HMC-1 cells co-cultured with U87 cells. HMC-1 cells (3 × 10^6 cells) and U87 cells (1 × 10^6 cells) were co-cultured for 30 or 60 min. Anti-CD40 Ab (300 ng/ml) or 8-oxo-dG (300 μg/ml) was pretreated 1 h and 30 min, respectively, before co-culture. CD40 siRNA was performed as described in “Materials and Methods”. A, The phosphorylation of PKC isoforms by each inhibitor in co-cultured HMC-1 cell. B, The phosphorylation of MAPKs by each inhibitor. Co-culture, co-cultured HMC-1 cells; Anti-CD40 Ab, anti-CD40 antibody pretreatment; CD40 siRNA, HMC-1 cells co-cultured with CD40 siRNA transfected-U87 cells; 8-oxo-dG, 8-hydroxydeoxyguanosine. *, Numbers below bands are the ratio of band density of each Rho family, PKC isoforms or MAPKs versus those of control and total protein. The data are representative of four independent experiments (n = 4).

Supplemental figure 7. CD40 protein expression by CD40 siRNA in U87 cells. Transfection was carried out according to the manufacture’s method. Vectors expressed CD40 siRNA or control siRNA in transfected-U87 cells.
Supplemental Figure 1
Supplemental Figure 2

HMC-1  U87  Co-culture

IL-6  RANTES  MCP-1  MIG  TNFα
Supplemental Figure 3
Supplemental Figure 4