Scavenger Receptor Collectin Placenta 1 (CL-P1) Predominantly Mediates Zymosan Phagocytosis by Human Vascular Endothelial Cells

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Collectin placenta 1 (CL-P1), a recently discovered scavenger receptor, mediates the uptake of oxidized low density lipoprotein and microbes. In this study, we investigated CL-P1-mediated binding and ingestion of yeast-derived zymosan bioparticles using Chinese hamster ovary (CHO) cells stably expressing human CL-P1 (CHO/CL-P1) and human vascular endothelial cells constitutively expressed CL-P1. The uptake of zymosan by CHO/CL-P1 was dependent upon the level of CL-P1 expressed on the membrane and was inhibited by cytochalasin D and wortmannin. The binding of zymosan was also inhibited by ligands of other scavenger receptors such as poly(I) and dextran sulfate. Real time reverse transcription-PCR analyses showed that other scavenger receptors, namely LOX-1, Stabilin-2, or macrophage receptor with collagenous structure (MARCO), were not expressed in human umbilical vein endothelial cells isolated from different individuals. Nonopsonic zymosan ingestion was inhibited in three primary cultured vascular endothelial cells, including different human umbilical vein endothelial cells from nine individuals treated with CL-P1 small interfering RNAs, although small interfering RNAs of other scavenger receptors had no effect on zymosan uptake in these cells. Furthermore, we confirmed that CL-P1 is expressed in human and murine vascular endothelial layers. Our results demonstrated that CL-P1 predominantly mediated phagocytosis for fungi in vascular endothelia.

Scavenger receptors are integral membrane proteins consisting at least eight different subclasses (Class A to Class H), with little amino acid sequence homology, that bind to a wide variety of ligands, including modified or oxidized low density lipoproteins (oxLDLs), apoptotic cells, and microbial pathogens (1). Among these ligands, oxLDLs are considered to have an important role in interactions with endothelial cells, macrophages, and smooth muscle cells in the development of atherosclerosis according to Ross’s response-to-injury hypothesis (2, 3). Vascular endothelial cells express several distinct scavenger receptors, such as SR-BI (4–6), LOX-1 (7), SREC (8), FEEL-1/stabilin-1, and FEEL-2/stabilin-2 (9).

Using placental cDNA, we recently identified CL-P1 (10), a type II transmembrane protein with a coiled-coil, a collagen-like domain, and a carbohydrate recognition domain (CRD) and showed it to be a scavenger receptor, in addition to its role as a collectin (Colec12). This molecule resembles a Class A scavenger receptor (SR-A) in its structure except for the replacement of the cysteine-rich domain by a CRD (11). CL-P1 can bind to oxLDL without interacting with other modified LDLs such as acetyl-LDL. Interestingly, we found that CL-P1 expression in HUVECs was up-regulated after the induction of oxidative stress in vitro and was increased in aortic endothelia in a rat ischemia-reperfusion model (12). Another reported finding on the ability of CL-P1 to mediate the binding of yeast, as well as Gram-negative and -positive bacteria in CL-P1-transfected CHO cells, strongly suggested a significant role for CL-P1 in host defense (10).

Phagocytosis in mammals is a process that involves the binding and internalization of pathogens and is essential for host defense. It is mainly achieved through the action of neutrophils, monocytes, and macrophages as “professional” phagocytes (13). The phagocytic process is mediated by a large number of different receptors that facilitate the attachment of particles on

2 The abbreviations used are: oxLDL, oxidized low density lipoprotein; LDL, low density lipoprotein; CL-P1, collectin placenta 1; LOX-1, lectin-like oxidized LDL receptor-1; SREC, scavenger receptor expressed by endothelial cells; FEEL-1, fascilin, epidermal growth factor-like, laminin-type epidermal growth factor-like, and link domain containing scavenger receptor-1; CRD, carbohydrate recognition domain; CR, complement receptor; CHO, Chinese hamster ovary; siRNA, small interfering RNA; HUVEC, human umbilical vein endothelial cell; Ab, antibody; mAb, monoclonal Ab; HUAEC, human umbilical artery endothelial cell; HAEC, human aortic endothelial cell.
the cell surface. These receptors may interact with targets, either directly through structural determinants present on the target surface (nonopsonic phagocytosis) or indirectly through opsonins supplied by the host (opsonin-dependent phagocytosis) (14). Currently, the well characterized opsonin receptors are Fcγ-receptor (FcγR) and complement receptor 3 (CR3), which bind to the Fc portion of IgG and complement iC3b, respectively (15).

In recent reports, Dectin-1, a type II transmembrane receptor containing a single extracellular C-type lectin domain, was shown to mediate phagocytosis of particles containing β-glucan, a major structural component of fungal cell walls. Dectin-1 is expressed widely in phagocytes, including macrophages and dendritic cells, and interacts with β-glucans (16, 17). Dectin-1 and Toll-like receptor 2 were reported to collaborate in coordinating inflammatory responses, such as cytokine secretion and the production of reactive oxygen species, in response to β-glucan-containing particles (18). Another novel C-type lectin, SIGN-R1, which is highly expressed in macrophages in the marginal zone of spleen, mediates the uptake of polysaccharides and encapsulated Streptococcus pneumoniae (19). Some scavenger receptors have also been shown to bind to various microbes, including bacteria and yeast. Of special note, studies on SR-A knock-out mice have revealed an enhanced sensitivity to both Staphylococcus aureus and Listeria infection. This suggested the important roles of SR-A scavenger receptor in innate immunity and in cross-talk with acquired immunity (20, 21).

In this study, we investigated CL-P1-mediated phagocytosis of zymosan by human vascular endothelial cells as well as by CHO/CL-P1, and we characterized its recognition in the phagocytic process. The role of CL-P1 in fungal phagocytosis in human vascular endothelia is discussed.

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents**—Ham’s F-12 medium was purchased from Sigma. Endothelial basal medium-2 (EBM-2) with bullet kit additives and primary human vascular endothelial cells were from Cambrex (La Jolla, CA). Texas Red-conjugated zymosan A bioparticles, rabbit anti-zymosan Ab, and Alexa Fluor 488-conjugated goat anti-rabbit IgG were from Invitrogen. Antibodies to CL-P1 were obtained using the recombinant CRD of human CL-P1 in Escherichia coli. Cytochalasin D and Wortmannin were from Calbiochem. Poly(I), poly(C), and dextran sulfate (500 kDa) were from Sigma. The preparation of oxidized LDL was carried out as described previously (10). Other chemicals, unless mentioned otherwise, were purchased from Sigma.

**Cell Culture**—CHO/Ida7 and CHO/CL-P1 cells were cultured as described previously (10). Three types of human primary endothelial cells were used. HUVECs (nine clones from different donors), human umbilical artery endothelial cells (HUAEC), and human aortic endothelial cells (HAEC) were maintained according to the supplier’s directions (Cambrex, La Jolla, CA). All experiments with HUVECs were performed between the third and sixth passages. In cases of HUAEC and HAEC, all experiments were performed between the fifth and eighth passages.

**Zymosan Phagocytosis by Vascular Endothelial Cells**

**RNA Isolation and Real Time RT-PCR Analysis**—Total RNA was prepared using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. The concentration of each RNA sample was measured using an Ultraview spectrophotometer (GE Healthcare). Only RNA samples with a 260/280 ratio (an indication of protein contamination) between 1.9 and 2.0 were used for the analysis. Reverse transcription (RT) reaction from 1 μg of RNA template was performed using TaqMan reverse transcriptase reagents (Applied Biosystems, Foster City, CA). A real time PCR was performed using an Applied Biosystems 7500 real time PCR system, and the collected data were analyzed with 7500 System SDS software, version 1.3, according to the manufacturer’s protocol. The threshold cycle (Ct) values for each transcript were compared with those for 18 S RNA, which remained relatively constant in abundance. Primers and probes used to confirm all transcripts were proprietary oligonucleotides obtained from TaqMan gene expression assays (Applied Biosystems) and utilized a 5′-FAM reporter and a 3′-nonfluorescent minor groove binder (supplemental Table 1). All PCRs were performed in 96-well optical reaction plates (Applied Biosystems, Foster City, CA) applying the following conditions: 2 min at 95 °C, 50 cycles of 15 s at 95 °C, and 1 min at 60 °C. Triplicate real time RT-PCR analyses were executed for each sample, and the obtained Ct values were averaged. Using the comparative Ct method described in the manual from Applied Biosystems, gene expression was normalized to the expression of the housekeeping gene 18 S rRNA, yielding the ΔCt value. The average ΔCt value obtained from the negative control was then subtracted from the average ΔCt value of each corresponding sample, yielding the ΔΔCt value. The gene expression level, normalized to that of the housekeeping gene, and relative to the control sample, was calculated using 2−ΔΔCt. Data were normalized to an untreated, negative control arbitrarily set at 1.

**siRNA Transfection**—CL-P1 siRNAs and other siRNAs were purchased as four oligonucleotide siGENOME SmartPools or ON-TARGETplus SmartPools (Dharmacon Research, Inc.), each containing about 50% guanidine-cytosine content (supplemental Table 2). The negative control sequence used was fluorescein siRNA having no perfect matches to known human genes (Dharmacon Research, Inc.). Lamin siRNA was commercially available as a SmartPool (Dharmacon Research, Inc.). HUVECs were trypsinized, washed with Hanks’ balanced salt solution, and resuspended (106 cells) in HUVEC solution (Amaxa Biosystems) containing 1–2 μM siRNA duplex, and were then transfected using a Nucleofector (Amaxa Biosystems) following the manufacturer’s instructions. The negative control siRNA-treated HUVECs were used as a mock transfection. After transfection, cells were immediately plated in collagen-coated dishes containing pre-warmed complete medium. After 48 h, cells were transferred to 35-mm collagen-coated dishes or collagen gel coverslips, and 24 h later, they were either lysed or subjected to phagocytosis assays, as described.

**Cell Lysis and Immunoblotting**—Cells were washed twice with phosphate-buffered saline and lysed in immunoprecipitation buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.25 mM EDTA (pH 8.0), 1% deoxycholic acid, 1% Triton X-100, 5 mM NaF, and 1 mM sodium orthovanadate supplemented with
FIGURE 1. Nonopsonic zymosan phagocytosis is dependent on the CL-P1 expression level. A, analysis of the surface CL-P1 expression level using anti-CL-P1 mAb with flow cytometry. Three clones of CHO/CL-P1 plus CHO-IdlA7 cells were tested. Cells were incubated with mouse anti-CL-P1 mAb and Alexa Fluor 488-conjugated goat anti-mouse IgG at 4 °C for 30 min and assayed with a FACSCalibur. B, simple method for determination of zymosan phagocytosis. The cells were incubated with Texas Red-conjugated zymosan particles for 2 h at 37 °C. After the cells were incubated with rabbit anti-zymosan Ab and Alexa Fluor 488-conjugated goat anti-rabbit IgG at 4 °C for 30 min and fixed with 4% paraformaldehyde, the cells were then visualized with the Olympus IX70-23FL/DIC-SP and SPOT2-SP system. Particles that were red color-positive but green color-negative were considered to be internalized. In the merged image, yellow particles indicate extracellular-adherent zymosan (white arrowhead), and red particles indicate intracellular zymosan (white arrow). Phagocytosed cells became larger in the phase contrast view. C, confocal microscopic image of zymosan phagocytosis obtained after the same procedures as in B. The yellow particles indicate extracellular-adherent zymosan, and red particles indicate intracellular zymosan (scale bars, 40 μm for all images). D, phagocytic index of CL-P1-transfected CHO cells. Phagocytosis was quantified using the total number of internalized zymosan particles (red particles) per 100 cells as the phagocytic index. Data are means ± S.D. of three experiments. A minimum of 200 cells was counted per experiment. **, p < 0.01.
protease inhibitor mixture (Roche Diagnostics) on ice for 10 min. Cell lysates were clarified by centrifugation at 15,000 rpm at 4 °C for 20 min, and protein concentrations were determined by the BCA method (Pierce). Equal amounts of cell lysates were incubated overnight at 4 °C with primary Ab. Immune complexes were collected at 4 °C for 3 h with protein G-Sepharose 4B (GE Healthcare). The precipitates were pelleted by centrifugation and washed three times with lysis buffer. The final pellets were boiled in SDS-PAGE sample buffer containing 10% β-mercaptoethanol and separated by SDS-PAGE. For immunoblot analysis, samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Co., Bedford, MA), and residual binding sites on the filters were blocked by incubation with Block Ace blocking buffer (Dainippon Sumitomo Pharma Co.) for 1 h at room temperature or overnight at 4 °C. The membranes were subsequently incubated with the appropriate antibodies and developed using an ECL (GE Healthcare). The results were visualized using an LAS-3000 (Fuji Photo Film Co.).

Binding and Phagocytosis Assays—For the zymosan binding assay, CHO/CL-P1 cells were incubated at 4 °C for 30 min with nonlabeled or Texas Red-conjugated zymosan A bioparticles. After binding, cells were washed and then fixed at room temperature for 20 min with 4% paraformaldehyde. Texas Red-conjugated zymosan A was the source of particles for phagocytosis assays. For most experiments, zymosan A was added at a final concentration of 10 particles per cell. All endothelial cells grown on collagen gel coverslips were overlaid with zymosan A. The cells were then incubated with the particles for 2 h at 37 °C. External particles were labeled in the cold with rabbit anti-zymosan Ab and Alexa Fluor 488-conjugated goat anti-rabbit IgG. Cells were fixed with 4% paraformaldehyde. Particles that were red color-positive but Alexa Fluor 488 (green color)-negative were considered to be internalized. Nuclear counterstaining was performed with Hoechst 33342 obtained from Invitrogen. To block the phagocytosis of zymosan particles, the transfected CHO/CL-P1 cells or HUVECs were preincubated for 30 min at 37 °C with the appropriate inhibitors diluted in serum-free Ham’s F-12 or EB M-2 basal medium prior to the addition of zymosan. We verified that the preincubation time and temperature were optimal to obtain an efficient blockade of phagocytosis. Fluorescent particles were visualized using the Olympus IX70-23FL/DIC-SP and SPOT2-SP system (Olympus Co. Ltd.). Phagocytosis was quantified using the total number of internalized zymosan particles per 100 cells as the phagocytic index (>1000 cells were counted). After the same staining, phagocytosed bioparticles were observed under an Olympus FLUOVIEW 1000 confocal laser-scanning microscope and analyzed using ASW software (version 3.1). All experiments were independently performed at least three times, and data are expressed as means ± S.D.

Flow Cytometry—Immunofluorescent flow cytometry was performed with several CHO/CL-P1 transfectants. Cells were incubated with mouse anti-CL-P1 mAb and Alexa Fluor 488-conjugated goat anti-mouse IgG at 4 °C for 30 min and assayed with a FACSCalibur flow cytometry system (BD Biosciences). Flow cytometry analyses showed that the x axis was fluorescence intensity (FL1-H) of CL-P1 and the y axis was cells counts.

Immunohistochemistry—Transgenic Tie-2-lacZ mice expressing β-galactosidase under the control of the endothelium-specific protein Tie-2 promoter were purchased from The Jackson Laboratory (Bar Harbor, ME). An endothelium-specific enhancer was introduced into the first intron of the mouse Tie-2 gene. A combination of the Tie-2 promoter with the intron fragment containing this enhancer allows for specific and uniform gene expression in virtually all vascular endothelial cells throughout embryogenesis and adulthood. Mice were anesthetized and perfused with 0.9% NaCl solution and subsequently perfusion-fixed with 4% formaldehyde (pH 7.2) for 2 and 10 min, respectively. Mouse hearts were collected and treated as described elsewhere (10). Specimens were dehydrated and embedded in paraffin. Immunohistochemistry was performed on 5-μm-thick paraffin sections using rabbit anti-CL-P1 Ab or anti-β-galactosidase mAb (Dako, Denmark), and anti-rabbit IgG conjugated with biotin (Vector Laboratories), and streptavidin conjugated with Alexa Fluor 488 (Invitrogen) or goat anti-mouse IgG conjugated with Alexa Fluor 594 (Invitrogen). In case of human heart, cryo-embedded sections

FIGURE 2. Binding of zymosan to CHO/CL-P1 cells. CHO/CL-P1 cells were permanent transfectants of CL-P1. Texas Red-labeled zymosan was incubated at 4 °C for 30 min with CHO/CL-P1. A–F, zymosan bound to the cell surface of CHO/CL-P1. The binding of zymosan was inhibited by dextran sulfate (B) and poly(I) (C) but not by poly(C) (D) or two phagocytic inhibitors of wortmannin and cytochalasin D (E and F). A is a nontreated control. Red spots are zymosan particles bound to the cell surface, and blue spots indicate nuclear counterstaining with Hoechst 43322 (scale bars, 100 μm for all images). G, zymosan binding was inhibited by scavenger receptor ligands and polyanionic substances but not by the phagocytic inhibitors. Poly(I), 10 μg/ml; poly(C), 10 μg/ml; dextran sulfate, 10 μg/ml; wortmannin, 200 nM; and cytochalasin D, 5 μM. Each bar indicates the relative binding percentage compared with the nontreated CHO/CL-P1 control. A minimum of 200 cells was counted per experiment. *, p < 0.01.
were used. Immunohistochemistry was performed on 10-μm-thick sections using rabbit anti-CL-P1 Ab or anti-CD31 mAb (Dako, Denmark), and anti-rabbit IgG conjugated with biotin (Vector Laboratories), and streptavidin conjugated with Alexa Fluor 488 or goat anti-mouse IgG conjugated with Alexa Fluor 594. Nuclear counterstaining was performed with TO-PRO-3 iodide obtained from Invitrogen. The fluorescent images were observed under an Olympus FLUOVIEW 1000 confocal laser-scanning microscope and analyzed using ASW software (version 3.1).

**Statistics**—Data are presented as the mean ± S.D. of the specified number of experiments performed in triplicate. The significance of difference was determined by a paired or unpaired Student’s *t* test. Unless otherwise stated, data are not significantly different from control values.

**RESULTS**

**Phagocytosis of Zymosan by CL-P1 cDNA-transfected CHO Cells**—We transfected human CL-P1 cDNA into CHO-IdaA7 cells (lacking functional LDL receptors) and chose three stable clones on the basis of the expression level of CL-P1 determined using flow cytometry (Fig. 1A). The phagocytic properties of CL-P1 cDNA-transfected CHO cells (CHO/CL-P1) were evaluated by quantifying the amount of internalized zymosan particles as red-colored dots using a fluorescent microscope (Fig. 1B). CHO/CL-P1 cells were incubated with Texas Red-conjugated zymosan particles for 2 h at 37 °C. Unbound zymosan particles were washed, and external zymosan particles were labeled in the cold (at 4 °C) with rabbit anti-zymosan Ab and Alexa Fluor 488-conjugated goat anti-rabbit IgG. Those zymosan particles that were Texas Red-positive but Alexa Fluor 488-negative were considered to be internalized. The phagocytic index was calculated from the number of internalized zymosan particles per 100 cells. Some phagocytosed cells became larger than others (Fig. 1B, **phase contrast view**). The binding and internalization of zymosan were also analyzed simultaneously by laser-confocal microscopy analysis (Fig. 1C). The amounts of attached and phagocytosed zymosan in these cells were dependent on the surface expression level of the scavenger receptor, CL-P1 (Fig. 1D). The binding of zymosan was inhibited by ligands of scavenger receptor and polyanionic substances (poly(I) and dextran sulfate) as in the case of oxLDL binding (10) (Fig. 2, A–C and G). Cytochalasin D, an antagonist of phagocytic inhibitors on nonopsonic zymosan phagocytosis by HUVECs.

**FIGURE 3. Effect of phagocytic inhibitors on nonopsonic zymosan phagocytosis.** A–C, cytochalasin D (CytoD) treatment inhibited CL-P1-mediated zymosan phagocytosis. D–F, Wortmannin treatment also inhibited CL-P1-mediated zymosan phagocytosis. CHO/CL-P1 cells were permanent transfectants of CL-P1. The cells were allowed to internalize zymosan for 2 h before analysis. A and D, each bar represents the relative degree of phagocytosis compared with the nontreated CHO/CL-P1 control. B and E represent the nontreated CHO/CL-P1 control. C and F represent phagocytic inhibitor-treated CHO/CL-P1. B and C, and E and F, yellow particles indicate extracellular adherent zymosan and red particles indicate intracellular zymosan (scale bars, 20 μm for all images). Each bar represents the percentage of phagocytosis compared with the nontreated CHO/CL-P1 control. The phagocytic index was 65.1 ± 7.3 in the negative control cells. Data are means ± S.D. of three experiments. A minimum of 200 cells was counted per experiment. **, *p* < 0.05; ***, *p* < 0.01.

**FIGURE 4. Nonopsonic zymosan phagocytosis in HUVECs.** A–F, representation of nonopsonic zymosan binding and phagocytosis in HUVECs is as follows: control, A; scavenger receptor ligands such as oxLDL, B; dextran sulfate (the same as poly(I)), C; poly(C), D; wortmannin, E; and cytochalasin D, F; Yellow particles are extracellular adherent zymosan; red particles are intracellular zymosans, and blue particles indicate nuclear counterstaining with Hoechst 33342 (scale bars, 100 μm for all images). G, relative quantification of nonopsonic zymosan phagocytosis by HUVECs. Poly(I), 10 μg/ml; poly(C), 10 μg/ml; dextran sulfate, 10 μg/ml; oxLDL, 50 μg/ml; wortmannin, 200 nM; cytochalasin D, 5 μM. Phagocytosis was quantified using the total number of internalized zymosan particles per 100 cells as the phagocytic index. Each bar represents the percentage of phagocytosis compared with the untreated HUVEC. The phagocytic index was 30.7 ± 11.2 in the negative control HUVEC (clone ZF-1899). Data are means ± S.D. of three experiments. A minimum of 1000 cells was counted per experiment. ***, *p* < 0.01.
of actin assembly, disturbed zymosan uptake (Fig. 3, A–C), and wortmannin, a phosphatidylinositol 3-kinase inhibitor, inhibited uptake mediated by CL-P1 (Fig. 3, D–F) similar to the phenomena seen in macrophages. In contrast, CL-P1-mediated binding of zymosan was not inhibited by treatment with wortmannin and cytochalasin D in CHO/CL-P1 (Fig. 2, E–G). The morphological change in cytochalasin D-treated cells caused by interference of cytoskeletal assembly did not affect the binding of zymosan (Fig. 3, C and F).

Scavenger Receptor-dependent Phagocytosis of Zymosan by HUVECs—Nonopsonic zymosan binding and ingestion by HUVECs were inhibited by the ligands of scavenger receptor, dextran sulfate, poly(I), and oxLDL (Fig. 4, A–D and G). Furthermore, treatment with either cytochalasin D or wortmannin inhibited nonopsonic zymosan phagocytosis but had no effect on zymosan binding (Fig. 4, E–G).

In addition to CL-P1, vascular endothelial cells are known to express several scavenger receptors such as SR-B1, LOX-1, SREC, FEEL-1/Stabilin-1, and FEEL-2/Stabilin-2. We quantified each scavenger receptor in primary cultured HUVECs with real time RT-PCR (Fig. 5A and Table 1). HUVECs mainly expressed CL-P1, SR-B1, and CD36 mRNAs. No expression of LOX-1 and FEEL-2/Stabilin-2 was observed in early passaged HUVECs, whereas LOX-1 was faintly detected in long passaged cells. In early passaged cells, levels of expression of SREC and FEEL-1/Stabilin-1 were also very low. These results suggest that CL-P1 is a major scavenger receptor in vascular endothelial cells. The expression of mRNAs encoding a variety of phagocytic receptors (CR1, CR2, CR3, and FcγRs) could not be detected or little in vitro cultivated HUVECs was detected by real time RT-PCR (Table 1). Although expressions of FcγRII and CD11c/CD18 (CR4) were detected, mRNA levels were about 1000 times lower than CL-P1. The mRNA of the neonatal Fc receptor (FcRn) known to modulate IgG transport in endothelial cells was detected three times higher than that of CL-P1.

Yeast Is a Natural Ligand for CL-P1 in Vascular Endothelial Cells—RNA interference studies with siRNAs specific to CL-P1 and other scavenger receptors were performed using human vascular endothelial cells. Immunoblotting showed a nearly complete reduction of CL-P1 in CL-P1 siRNA-treated HUVECs (Fig. 5C) but no change in the expression of other scavenger receptors, several angiogenic factors, and their receptors (Fig. 5B), nor did the knockdown of

**FIGURE 5. Depletion of scavenger receptor CL-P1 by siRNAs.** A, relative quantification of the mRNA levels of endothelial scavenger receptors and C-type lectins in primary HUVECs. LOX-1, Stabilin-2/FEEL-2, and dendritic-cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN) could not be detected in our experimental system. B, change of the mRNA level of endothelial scavenger receptors, angiogenesis-related molecules, and their receptors in HUVECs after CL-P1 siRNA transfection. Duplicate real time RT-PCR analyses were carried out for each sample, and relative expressions from the obtained Ct values were averaged for nine HUVEC clones. The CL-P1 siRNA treatment was performed with an electroporation method. The cells were grown for 48 h, transferred to a 35-mm collagen-coated dish and collagen gel coverslip, and further grown for 24 h. They were then lysed for RNA purification. C, immunoblotting of CL-P1 protein in HUVECs after siRNA transfection. The three representative HUVEC clones used were from different donors. Cell lysates were immunoprecipitated with anti-CL-P1 mAb, and immunoblotting analysis was performed with rabbit anti-CL-P1 Ab. Protein expression was decreased, and no band could be detected by immunoblotting (black arrow, glycosylated CL-P1; black arrowhead, insufficiently glycosylated CL-P1; asterisk, nonspecific band). Lane 1, negative control siRNA; lane 2, CL-P1 siRNA (mixture of four kinds of CL-P1 siRNA); lane 3, CL-P1 siRNA (No. 4).
CL-P1 cause changes in the growth and morphology of endothelial cells (data not shown). CL-P1 protein on the Western blots had two bands of 140 and/or 110 kDa, the lower one probably being under-glycosylated (10). SR-B1 and SREC siRNA treatment induced partial cell growth inhibition. Treatment with the control siRNA did not cause any changes in HUVECs. We showed that the depletion of CL-P1 specifically inhibited zymosan binding and phagocytosis in HUVECs from individuals (n = 9) by over 50% (Fig. 6, A and B, and supplemental Fig. 1). Furthermore, we demonstrated that zymosan binding and phagocytosis were inhibited in two other primary cultured endothelial cells, namely HUAEC and HAEC treated with CL-P1 siRNA, as they were in HUVECs (Fig. 6C). The treatment with siRNA of other scavenger receptors did not affect zymosan phagocytosis in HUVECs (Fig. 7, A and B). These results imply that CL-P1 plays a significant role in zymosan phagocytosis by human vascular endothelial cells and that fungi may be a natural CL-P1 target.

CL-P1 Was Mainly Expressed in Vascular Endothelial Cells of Humans and Mice—In previous studies, we detected CL-P1 mRNA expression in various tissues but not in macrophages, monocytes, or hepatic Kupffer cells (10). An immunohistochemical analysis in mice showed that CL-P1 was localized by tube-like structures of the heart (10). In this study, we investigated the localization of CL-P1 to find its expression in most vascular endothelial layers, of tie-2 promoter gal transgenic mice. tie-2 promoter gal transgenic mice expressed β-galactosidase as a reporter gene under the control of the endothelium-specific Tie-2 promoter in endothelial layers. CL-P1 was co-localized with β-galactosidase-positive vascular endothelial layers in murine heart (Fig. 8, A and B). We also detected expression of CL-P1 in human heart cryo-sections (Fig. 8, C and D). CL-P1-positive cells were found among CD31-positive endothelial cells by immunohistochemical analyses. CL-P1 expression was also found in smooth muscle layers in these cases, but the mRNA and protein level of CL-P1 in aortic smooth muscle cells were lower than in aortic endothelial cells (HAECs) or umbilical endothelial cells (HUVECs and HUAECs) (data not shown). Therefore, we confirmed that CL-P1 was generally expressed in vascular endothelial layers in both humans and mice.

DISCUSSION
Phagocytosis is a process by which cells take up relatively large particles (>0.5 μm) into their vacuoles, contributing to a central mechanism in tissue remodeling, inflammation, and defense against infectious molecules (22). In this study we report for the first time which receptor mainly mediated zymosan phagocytosis in endothelial cells through siRNA experiments. The reduction in zymosan binding and phagocytosis seen in HUVECs and in the other two human primary cultured
vascular endothelial cells, which were associated with the down-regulation of CL-P1 mRNA and protein in our siRNA study, indicates the central role of CL-P1 in these two processes. Furthermore, the nonopsonic phagocytosis of zymosan in both CHO/CL-P1 cells and HUVECs was very similar to IgG-opsonized phagocytosis in macrophages in that it was inhibited by wortmannin and cytochalasin D (Figs. 3 and 4). In addition, the interaction between CL-P1 and zymosan stimulates the spreading of pseudopods around the particles through a process that seems like a “zipper mechanism” between Fcγ receptor and opsonized IgG.

HUVECs took up nonspecific IgG-opsonized zymosan and nonopsonized zymosan to a similar extent, but they failed to bind or ingest the zymosan-specific IgG-opsonized zymosan (supplemental Fig. 2). Opsonization with zymosan-specific IgG might ensure coverage of the zymosan so that the attachment between ligands of the zymosan particle and cell surface receptors, CL-P1, would seem to be partially suppressed. Furthermore, the treatment with siRNA of opsonin receptors did not affect serum-opsonized zymosan phagocytosis in HUVEC (supplemental Fig. 3). The inhibitory effect on zymosan phagocytosis by CL-P1 siRNA-treated experiments was not coinci-

dent with the expression level of CL-P1 protein in HUVECs. Although we tried to demonstrate whether other scavenger receptors or opsonic receptors act as the candidates of zymosan phagocytosis or not, we failed to find out other known candidate receptors (Fig. 7 and supplemental Fig. 3). These results imply the existence of unknown scavenger receptors or opsonic receptors involved in the zymosan phagocytosis by endothelial cells. Moreover, the zymosan binding to CHO/CL-P1 inhibited by polyanionic molecules was almost complete, but the binding to HUVECs was partial. These results suggest that the binding between CL-P1 and zymosan is dependent on electrostatic charges, and there might be an unknown phagocytic receptor in these endothelial cells. Although mRNA expression of FcRn was higher than that of CL-P1 in nascent HUVEC (Table 1), FcRn-targeted siRNA had no effect on zymosan phagocytosis.

The C-type lectin domain of CL-P1 has been shown to contain QPD tri-amino acid sequences as a lectin motif, which was expected to bind to α-galactose and GalNAc. In fact, CL-P1 expressed in 293 cells was shown to have affinity to Gal-type ligands and T and Tn antigen and to uptake GalNAc-conjugated particles in a Ca²⁺-dependent process (23). In addition, CL-P1 expression on vascular endothelial cells parallels that of selectin, which binds to Lewis X tri-saccharides bearing sialic
acid and sulfate groups. The unique binding to a specific glycan structure suggested that CL-P1 might participate in cell-cell adhesion in the form of leukocyte-endothelial cell interactions mediated by selectins (24–26). Another report showed that CL-P1 binds selectively to asialo-orosomucoid because of its high affinity binding to glycans bearing the Lewis X epitope (25).

Interestingly, human CL-P1 interacted directly with pathogenic microbes and caused phagocytosis in vascular endothelial cells. In general, signaling pathways involving FcRs and CRs in the phagocytic process lead to the activation of small GTP-binding proteins belonging to the Rho family (27). Rho proteins near the plasma membrane have a central role in the cytoskeletal remodeling during particle internalization. However, the signaling pathways triggered are distinct and depend on the phagocytic receptors engaged. In turn, each receptor activates distinct Rho proteins operating within different mechanisms for particle ingestion. We found that actin filaments extended outward close to the surface of zymosan-internalized CHO/CL-P1 cells, and some cells became giant cells (Fig. 1B). The morphology of CHO/CL-P1 cells and vascular endothelial cells treated with cytochalasin D was altered to a rounded form as the result of interference in the cytoskeletal assembly (Fig. 3C) (28–31). Phagocytosis driven by the acute assembly of F-actin is strictly dependent upon the cytoskeleton and does not require assembly of clathrin at sites of receptor clustering, yet is not strictly dependent upon the cytoskeleton and does not suggest Class I phosphatidylinositol 3-kinase activity (31). The strong inhibition of phagocytosis by wortmannin does not suggest signaling through CL-P1 could cause the activation of GTPase and induce the rearrangement of actin filaments.

Zymosan injection of experimental animals is known to produce a severe inflammatory response. Among various inflammatory mediators, zymosan and β-glucan induced macrophage to secrete interleukin-6, interleukin-8, tumor necrosis factor-α, and interleukin-1β (32–34). During the phagocytosis of opsonized zymosan by HUVECs, cytokine and oxygen radical production was observed in these cells (35). Cytokine-activated human vascular endothelial cells and dermal nurse-like cells, such as “nonprofessional” antigen presenting cells, play roles in initiating immune responses by interacting with immunocompetent cells via their Class II major histocompatibility complex molecules (36, 37). Although human endothelial cells do not express B7, they can support proliferation by antigen-specific T cell clones (38, 39).

Finally, we showed the involvement of CL-P1 in nonopsonic zymosan phagocytosis through siRNA studies of other vascular expressed scavenger receptors and opsonin receptors using primary cultured human vascular endothelial cells. Furthermore, the localization of CL-P1 in human tissues was confirmed by immunohistochemical analyses. In our study of phagocytosis by vascular endothelial cells as a primitive innate immune function, we demonstrated the predominant role of the scavenger receptor CL-P1 in the recognition and phagocytosis of fungi.

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