C-Reactive Protein Uptake by Macrophage Cell Line via Class-A Scavenger Receptor

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BACKGROUND: C-reactive protein (CRP) increases in response to inflammation and is purported to be a risk factor for atherogenesis. We recently demonstrated that a scavenger receptor, lectin-like oxidized LDL receptor (LOX-1), is a receptor for CRP. In light of the overlapping ligand spectrum of scavenger receptors such as modified LDL, bacteria, and advanced glycation end products, we examined whether other scavenger receptors recognize CRP.

METHODS: We analyzed the uptake of fluorescently labeled CRP in COS-7 cells expressing a series of scavenger receptors and in a monocytic cell line, THP-1, differentiated into macrophage with phorbol 12-myristate 13-acetate (PMA). We applied small interfering RNA (siRNA) against class-A scavenger receptor (SR-A) to THP-1 cells to suppress the expression of SR-A. We also analyzed the binding of nonlabeled CRP to immobilized recombinant LOX-1 and SR-A in vitro using anti-CRP antibody.

RESULTS: COS-7 cells expressing LOX-1 and SR-A internalized fluorescently labeled CRP in a dose-dependent manner, but cells expressing CD36, SR-BI, or CD68 did not. The recombinant LOX-1 and SR-A proteins recognized nonlabeled purified CRP and native CRP in serum in vitro. THP-1 cells differentiated into macrophage-like cells by treatment with PMA internalized fluorescently labeled CRP. siRNA against SR-A significantly and concomitantly inhibited the expression of SR-A (P < 0.01) and CRP uptake (P < 0.01), whereas control siRNA did not.

CONCLUSIONS: CRP is recognized by SR-A as well as LOX-1 and taken up via SR-A in a macrophage-like cell line. This process might be of significance in the pathogenesis of atherosclerotic disease.

1 Nonstandard abbreviations: CRP, C-reactive protein; LOX-1, lectin-like oxidized LDL receptor; SR, scavenger receptor; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; siRNA, small interfering RNA; PMA, phorbol 12-myristate 13-acetate; SRA-C6, anti-SR-A antibody; DAPI, 4’,6-diamidino-2-phenylindole.
dectin-1 (GenBank NM197947), which were subcloned into pcDNA6.2/V5/GW/D-TOPO expression vector (Invitrogen). We used pcDNA3.1/V5-His/lacZ (Invitrogen) as a control. After 48 h, we washed the cells with DMEM:1% antibiotics and antimycotic (AbAm; Invitrogen). We replaced the medium with CypHer5E-CRP-containing DMEM:1% AbAm and incubated the cells for 2 h at 37 °C. After washing with PBS, the cells were fixed with phosphate-buffered formalin (Wako) and permeabilized with 0.1% Triton X-100/PBS. We detected the expression of each receptor by immunostaining with anti-V5 antibody (Nacalai Tesque) combined with Alexa 488 antitmouse IgG (Invitrogen). The nuclei of the cells were counterstained with 0.5 mg/L 4′,6-diamidino-2-phenylindole (DAPI) (Sigma). We divided the fluorescence intensities of CypHer5E and Alexa 488 by the cell number in a field, then divided the CypHer5E-CRP fluorescence intensity in the field by the Alexa 488 fluorescence value. These quantitative analyses were performed with an IN Cell Analyzer 1000 system (GE Healthcare).

We prepared recombinant human SR-A (amino acids 76–358) as described for LOX-1 (10). Recombinant human SR-A (0.1 μg) or BSA (0.1 μg, Sigma) was immobilized to each well of 384-well plates (HighBind; Corning) by incubating at 4 °C in PBS overnight. After 2 washes with PBS, the plates were blocked with 80 μL of 20% ImmunoBlock (DS Pharma)/PBS at 4 °C for 8 h. After washing twice with PBS, we added CRP in the reaction buffer (10 mmol/L HEPES, 150 mmol/L NaCl, 2 mmol/L CaCl2, 1% BSA, pH 7.0) to each well and incubated them at 4 °C overnight.

We detected the binding of CRP with a TMB Peroxidase EIA Substrate kit (Bio-Rad) as described for LOX-1 (10). We obtained small interfering RNA (siRNA) duplex oligoribonucleotides targeting the SR-A coding region (GenBank NM002445) from Invitrogen and used stealth RNAi duplex (Invitrogen) as a negative control. The siRNA sequences were as follows: 5′-GAUAUAACUAAAGUCUCACGGGAA-3′, 5′-UCCCGUGAGACUUUGAGUUAUC-3′, and 5′-CAGACCUGAGAAUACUCUUA-3′, 5′-UUAAGUGAUAAUUCUCAGGUCUG-3′.

THP-1 cells were maintained with 10% FBS/1% AbAm/20 μmol/L mercaptoethanol:RPMI 1640 and differentiated with 100 nmol/L phorbol 12-myristate 13-acetate (PMA) (Sigma) for 48 h. We transfected the cells with siRNA oligos or control siRNA using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer’s instruction. After incubation at 37 °C for 24 h, we washed the cells with RPMI 1640/1% AbAm and replaced the medium with CypHer5E-CRP-containing RPMI 1640/1% AbAm, and the cells were incubated for 2 h. After washing with PBS, the cells were fixed with phosphate-buffered formalin (Wako) and permeabilized with 0.1% Triton X-100/PBS. We detected the effects of downregulation of SR-A gene expression by immunostaining with anti-SR-A antibody (SRA-C6; Trans Genic Inc) combined with Alexa 488 antitmouse IgG. For detection of Fcγ receptors, we used anti-CD32 antibody (AT10; Santa Cruz) and anti-CD64 antibody (10.1; Santa Cruz). For CRP detection, we used anti-CRP antibody (Bethyl). The nuclei of the cells were counterstained with 0.5 mg/L DAPI. We divided the fluorescence intensities of CypHer5E and Alexa 488 by the cell number in a field.

Quantitative analysis was performed with an IN Cell Analyzer 1000 system. All transfections were performed in triplicate.

All data are presented as mean (SE). Statistical analysis was performed with Student t-test. A P value <0.05 was considered statistically significant.

We examined whether CRP binds to scavenger receptors: LOX-1, SR-A, CD36, SR-BI, CD68, and A56-356-labeled CRP at the concentration of 1 mg/L at 4 °C bound significantly to LOX-1–expressing cells (P < 0.01) but bound poorly to the cells expressing the other receptors (Supplemental Fig. 1, which accompanies the online version of this article at www.clinchem.org/content/vol56/issue3). Cellular uptake of CypHer5E-labeled CRP, which shows fluorescence after endocytosis, was significantly higher in SR-A–expressing cells, in a dose-dependent manner (1–30 mg/L), as well as in LOX-1–expressing cells, compared with cells expressing the other receptors (Fig. 1). Immunostaining with anti-V5 antibody revealed that all the receptors were expressed at a similar level in the respective cells.

Using anti-CRP antibody, we confirmed that nonlabeled CRP was also taken up by SR-A–expressing COS-7 cells. We further observed a significant binding of nonlabeled CRP (0.1–1 mg/L) to immobilized recombinant SR-A (P < 0.01) (online Supplemental Fig. 2). The binding was not affected by polymyxin B (5 mg/L), suggesting that it did not depend on the presence of endotoxin. Importantly, native CRP contained in human serum showed significant binding to SR-A, as well as to LOX-1 (P < 0.01) (online Supplemental Fig. 3). The binding was dependent on the concentration of CRP in the serum, suggesting that SR-A and LOX-1 have a capacity to bind to a native form of CRP in serum in the presence of other plasma proteins. These results indicate that SR-A and LOX-1 are the receptors for CRP among the examined receptors.

Because SR-A works in the monocyte-macrophage system, we assessed whether CRP is taken up by macrophages via SR-A. We used a human monocytic cell line,
work in the cells stimulated by different molecules.

Fc family overlaps considerably macrophage cell line, at least under these conditions. indicating that CRP is taken up mainly via SR-A in the unidentified CRP receptor. Fc promoted uptake of oxidized LDL that fucoidin, a ligand for SR-A, inhibits the in vivo CRP- 
sisexpression of Fc is strongly induced, whereas the expres-

siRNA (Table 1). The siRNA targeting SR-A did not affect the expression of Fc receptors (data not shown), indicating that CRP is taken up mainly via SR-A in a macrophage cell line, at least under these conditions. The ligand specificity of the scavenger receptor family overlaps considerably (11, 12 ), and while all can bind to oxidized LDL, only SR-A or LOX-1 bound to CRP. Interestingly, dectin-1, the most structurally similar molecule to LOX-1, did not bind to CRP.

Using a monoclonal antibody, a previous report suggested the presence of an unknown receptor other than Fcγ receptors in macrophages (14). It has been reported that fucoidin, a ligand for SR-A, inhibits the in vivo CRP-promoted uptake of oxidized LDL (15). SR-A might be the unidentified CRP receptor. Fcγ receptors and SR-A are under different regulation of gene expression. In fact, in response to differentiation stimulus of PMA, the expression of SR-A is strongly induced, whereas the expression of Fcγ receptors is suppressed (16, 17). Conversely, stimulation by interferon-γ enhances the expression of Fcγ receptors but suppresses the expression of SR-A (18). These results suggest that Fcγ receptors and SR-A would work in the cells stimulated by different molecules.

Interestingly, the activity of SR-A as CRP receptor was more pronounced in the uptake of CRP, whereas LOX-1 showed strong activity in both binding and uptake. Because SR-A works in phagocytes, the CRP uptake activity of SR-A is reasonable. CRP was originally identified as a binding protein for bacterial component C-polysaccharide (3). SR-A may function to engulf bacteria, viruses, and harmful substances opsonized by CRP in a context of innate immunity.

Related to epidemiological risk factors for cardiovascular disease, the presence of CRP in atheroma has been reported in both rabbits and humans (6). Furthermore, the colocalization of CRP and SR-A in macrophages in atheromas has been reported (19). Taking these reports together with the present results, SR-A-mediated CRP uptake by macrophages in atheromas might affect the foam cell formation and progression of atherosclerotic disease.

**Table 1. Suppression of the uptake of fluorescently labeled CRP by siRNA against SR-A in differenti
ted THP-1 cells.**

<table>
<thead>
<tr>
<th>siRNA</th>
<th>SR-A expression, %</th>
<th>CypHer5E-CRP, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>103 (3.2)</td>
<td>108 (9.1)</td>
</tr>
<tr>
<td>Control siRNA</td>
<td>100 (2.7)</td>
<td>100 (9.8)</td>
</tr>
<tr>
<td>siRNA1 for SR-A</td>
<td>17 (1.6)b</td>
<td>31 (3.9)b</td>
</tr>
<tr>
<td>siRNA2 for SR-A</td>
<td>13 (0.0)b</td>
<td>32 (1.1)b</td>
</tr>
</tbody>
</table>

* Data are as mean (SE).

b Significant difference vs control siRNA groups (P < 0.01).

Fig. 1. Quantitative analyses of CypHer5E-CRP taken up by COS-7 cells expressing LOX-1, SR-A, CD36, SR-

BI, and dectin-1. Signals observed in the cells transfected with pcDNA3.1/ V5-His/1acZ were considered as non-specific background.

*Significant difference vs. negative control (P < 0.01).

THP-1, after inducing differentiation into macro-

phage by the treatment of PMA (13). In PMA-treated

THP-1 cells, CypHer5E-CRP was taken up in a dose-
dependent manner (0.3–30 mg/L). SR-A expression and CRP uptake were concomitantly suppressed by 2 different siRNAs targeting SR-A, but not by control siRNA (Table 1). The siRNA targeting SR-A did not affect the expression of Fcγ receptors (data not shown), indicating that CRP is taken up mainly via SR-A in a macrophage cell line, at least under these conditions.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

Authors’ Disclosures of Potential Conflicts of Interest: Upon manuscript submission, all authors completed the Disclosures of Potential Conflict of Interest form. Potential conflicts of interest:

Employment or Leadership: None declared.

Consultant or Advisory Role: None declared.

Stock Ownership: None declared.

Honoraria: None declared.

Research Funding: This study was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan; the Ministry of Health, Labour and Welfare of Japan; the National Institute of Biomedical Innovation; and Japan Science and Technology Agency.

Expert Testimony: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.
References