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Binding between Azurocidin and Calreticulin: Its Involvement in the Activation of Peripheral Monocytes

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We found that azurocidin, a secretory protein in neutrophils, binds to calreticulin, a multifunctional chaperone of the endoplasmic reticulum. Azurocidin is known to induce cytokine production in monocytes, but the mechanism of monocyte activation by azurocidin remains unknown. On the other hand, an antibacterial peptide, KLKL-LLLLKLK-NH_2 (L5), is known to bind to cell surface calreticulin of human neutrophils, resulting in their activation to produce O_2^-. Therefore, we examined whether cell surface calreticulin is involved in the activation of human monocytes by azurocidin to produce IL-6. We found that carlreticulin is in fact located on the surface of monocytes and that the IL-6 production stimulated by an azurucidin is inhibited by anti-calreticulin antibody. Possibly, binding between cell surface calreticulin and azurocidin is prerequisite for the activation of monocytes by azurocidin to produce IL-6.

Key words: azurocidin, calreticulin, monocyte, IL-6.

Abbreviations: IL-6, interleukin-6; O_2^-, superoxide anion; PtdIns, phosphatidylinositol.

Human calreticulin consists of 400 amino acid residues comprising three structural domains. The N-domain (residues 1–180) is the most conserved domain among calreticulins from various organisms. The P-domain (residues 181–291) is rich in proline, and the C-domain (residues 292–400) is very acidic. Calcium binds to the C-domain and an endoplasmic reticulum retrieval sequence, KDEL, is located at the end of this domain (*1*).

Calreticulin is assumed to be a molecular chaperone for MHC class I antigens (*2–6*). Contrary to its intracellular functions, several lines of evidence suggest it has extracellular functions related to signal transduction (*2*, *7–11*). Previously, we demonstrated that calreticulin is expressed on the surface of human neutrophils and functions as a receptor for an antibacterial peptide, L5, which exhibits affinity to calreticulin (*12*, *13*). Namely, on L5 binding to calreticulin expressed on the surface of human peripheral neutrophils or retinoic acid-activated U937 cells, the cells are activated in some way, and their production of O_2^- is found to be greatly enhanced. Treatment of the cells with anti-calretriculin antibody represses O_2^- production in the presence of L5. Moreover, as the enhancement of O_2^- production by L5 is selectively inhibited by pertussis toxin (PTX), we proposed a functional link between cell surface calreticulin and G-protein (Gi). However, as calreticulin contains no appreciable transmembrane domain, the presence of another membrane protein that mediates the interaction between cell surface calreticulin and Gi was predicted.

To identify such a protein, we intended to isolate U937 membrane proteins that bind to calreticulin, assuming that the mediator protein should be included among these calreticulin binding proteins. During this study, we incidentally found that azurocidin, a neutrophil secretory protein, has the ability to bind to calreticulin. In this report, we suggest that azurocidin induces human monocytes via cell surface calreticulin to generate IL-6. This mechanism seems to be essentially the same as the activation of neutrophils by the L5 peptide.

EXPERIMENTAL PROCEDURES

Antibodies—The anti-calreticulin N-domain and C-domain peptide antibodies used here were the same as described previously (*12*). Antibodies were raised against peptides corresponding to amino acid residues 54–73 of the N-domain, and 399–417 of the C-domain of human calreticulin.

Preparation of the Membrane Fraction—U937 cells were inoculated into RPMI 1640 containing 10% fetal bovine serum and 1 μM retinoic acid at a density of 2–3 × 10^5 cells/ml, and then cultured for 3 days. The cells were harvested and the membrane fraction was prepared as described previously (*14*). The resulting membranes (2.0 mg/ml of protein) were suspended in Hepes buffered saline [10 mM Hepes/NaOH (pH 7.4), 150 mM NaCl, 5 mM KCl, 1 mM EGTA] with 1% (v/v) Triton X-100, and the membrane proteins were solubilized. Debris was removed by centrifugation at 100,000 ×*g* for 30 min at 4°C, and the supernatant was defined as the detergent-

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soluble extract. Protein concentrations were determined by the method of Lowry *et al.* (*15*).

Preparation of Recombinant Calreticulins—Recombinant full-length, and N- (residues 1–182), P- (residues 182–290), and C-domain (residues 330–401) rabbit calreticulins were each expressed in *Escherichia coli* as a GST-fusion protein and purified as described previously (*16*, *17*). The cDNA and vector construct of rabbit calreticulin were generous gifts from Dr. Marek Michalak (Canadian Institutes of Health Research Group in Molecular Biology of Membranes and the Department of Biochemistry, University of Alberta).

Detection of Calreticulin-Binding Proteins in the Detergent-Soluble Extract of the U937 Membrane Fraction—The detergent-soluble extract of the U937 membrane fraction was subjected to affinity chromatography as described by Baksh *et al.* (*18*) to isolate proteins exhibiting affinity to calreticulin. Recombinant calreticulin (2 mg) was conjugated with 1 ml of CNBr-activated Sepharose 4B (Amercham Pharmacia Biotech, Uppsala, Sweden). The detergent soluble extract (3.2 mg/ml, 10 ml) was first applied to a pre-column to remove any proteins that associate nonspecifically with the column matrix, and then the resulting flow-through fraction was applied to the affinity column. The column volume was 1 ml. The column was washed with 15 ml of a solution comprising Hepes buffered saline (pH 7.4) with 0.1% Triton X-100, and then the bound proteins were eluted with the same buffer containing 1 M KCl. The eluted proteins were subjected to SDS polyacrylamide gel electrophoresis and stained with silver using 2D-silver-stain-II™ DAIICHI (Daiichi Pure Chemicals, Tokyo).

Binding between Azurocidin and Calreticulin—Recombinant rabbit calreticulin, its P-domain and C-domain were individually conjugated with CNBr-activated Sepharose 4B beads (Amersham Pharmacia Biotech, Uppsala, Sweden) as previously described (*18*). Azurocidin (10 μg) was mixed with 1 ml of Sepharose 4B beads, which retain about 2 mg of recombinant protein, and the mixture was kept overnight at 4°C with gentle shaking. The mixture was then centrifuged for 3 min at 2,000 ×*g* to separate the beads and the supernatant. The bound azurocidin was eluted from the beads by heating at 75°C for 20 min in electrophoresis buffer (50 mM Tris-HCl, pH6.8, 2% SDS, 6% 2-mercaptoethanol, 10% glycerol, 1% bromophenol-blue). Azurocidin recovered from the beads was analyzed by SDS polyacrylamide gel electrophoresis. For the reaction kinetics study, a plasmon resonance approach was performed in a BIAcore 2000 biosensor (Amercham Pharmacia Biotech, Uppsala, Sweden) (*19*). Recombinant human full-length calreticulin cDNA and vector construct were generous gifts from Dr Shunji Natsuka (Department of Applied Biology, Kyoto Institute of Technology). Recombinant calreticulin was coupled to carboxymethylated dextran sulfate (Sensor chip CM5; Amercham Pharmacia Biotech, Uppsala, Sweden) through the free thiol group (*20*). A purified azurocidin dissolved in Hepes buffered saline was subjected to analysis. The resonance signals were obtained at 25°C, as recommended by the manufacturer.

Activation of Human Monocytes by Azurocidin—Human monocytes (2×10^5 cells per well) were cultured in 24-well plates (Corning, NA, USA) and treated with 10 μg/ml of azurocidin or 10 μg/ml of bovine serum albumin (BSA), as a negative control, for 24 h in 500 μl of Dulbecco's modified Eagle's medium containing antibiotics (10 μg/ml polymixin B, 100 U/ml penicillin G, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B). Then the production of IL-6 in the medium was assayed using a Quantikine® human interleukin-6 ELISA system (R&D Systems, Minneapolis, MN, USA). To determine the effect of anti-calreticulin N-domain and C-domain peptide antibodies on the production of IL-6, 50 μg/ml of each antibody was added prior to the addition of azurocidin.

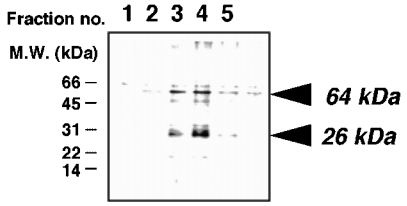

Fig. 1. **Electrophoretic profile of calreticulin binding proteins.** The detergent-soluble extract of the U937 membrane fraction was subjected to affinity column chromatography. Each fraction from the column was analyzed by SDS polyacrylamide gel electrophoresis, and the gel was stained with silver. The gel was calibrated with serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21 kDa), and lysozyme (14 kDa). The arrows indicate the positions of 64 kDa and 26 kDa proteins.

Indirect Immunofluorescence Study—Human monocytes (106) were treated with 10 μg/ml of anti-calreticulin C-domain peptide antibody or nonimmune IgG for 40 min at 4°C, washed three times with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 1,5 mM KH2PO4, 8 mM Na2HPO4, pH 7.5) containing 1% BSA, and then treated with fluorescent isothiocyanate-conjugated anti-(rabbit IgG) for 40 min at 4°C. The cells were then fixed with 4% paraformaldehyde, mounted, and examined under an Olympus BH2-RFCA fluorescence microscopic system (Olympus, Tokyo).

PtdIns-Specific Phospholipase C Treatment—Human monocytes (10^6) were suspended in 100 μl of 10 mM Hepes/NaOH (pH 7.4), 150 mM NaCl, and 1 mM EGTA, and treated with 400 mU (20 μl) of *Bacillus thuringiensis* PtdIns-specific phospholipase C (Funakoshi, Tokyo) for 60 min at 37°C. Then, the cells were collected and subjected to indirect immunofluorescence analysis.

Others—Human peripheral monocytes were isolated essentially as described by Boyum (*14*). Human azurocidin and myeloblastin were purchased from Athens Research and Technology (Athens, GA, USA).

RESULTS

Calreticulin Binding Proteins in the Detergent-Soluble Extract of the U937 Membrane Fraction—To identify the membrane proteins that interact with cell surface calreticulin, we performed calreticulin affinity column chromatography using the detergent-soluble extract of the U937 membrane fraction. As shown in Fig. 1, two major protein bands were detected when the fractions eluted from the column were subjected to SDS polyacrylamide gel electrophoresis. The protein with a molecular mass of 64 kDa

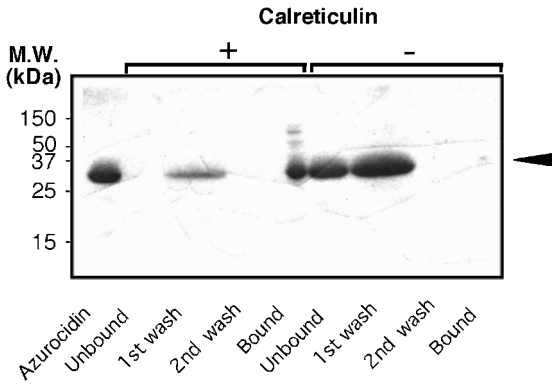

Fig. 2. **Binding between azurocidin and recombinant calreticulin.** Azurocidin was treated with calreticulin-coated Sepharose 4B beads. The beads were collected by centrifugation and washed twice, then the bound azurocidin was analyzed by SDS polyacrylamide gel electrophoresis, and the gel was stained with Coumassie Brilliant Blue. Unbound, supernatant fraction; 1st washed, first wash fraction; 2nd washed, second wash fraction; Bound, calreticulin bead-bound fraction. The gel was calibrated with precission protein standards (Bio-Rad, CA, USA). The arrow indicates the position of azurocidin.

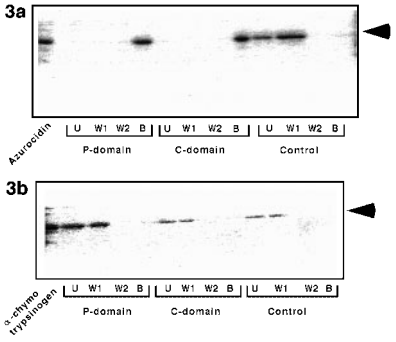

Fig. 3. **Binding between azurocidin, and the P and C-domains of calreticulin.** The binding of calreticulin or α-chymotrypsinogen to the recombinant P or C-domain of calreticulin was examined as described in the legend to Fig. 1. (a) Azurocidin, (b) α-chymotrypsinogen as a negative control. The arrows indicate the positions of the respective proteins.

was identified as a protein disulfide isomerase from its N-terminal amino acid sequence. This enzyme is known to be located in the endoplasmic reticulum and to exhibit affinity for calreticulin (*18*). The other protein, with a molecular mass of 26 kDa, was found to be a mixture of azurocidin and myeloblastin, which are closely related neutrophil secretory proteins belonging to the serine protease family (*21*). Contrary to our expectation, these are not membrane proteins that mediate the interaction between cell surface calreticulin and Gi.

However, as nothing was known about the binding between the two secretory proteins (azurocidin and myeloblastin) and calreticulin, we examined the binding further, assuming that their interaction with cell-surface calreticulin is biologically significant. First, we confirmed their binding to calreticulin. For this, we incubated calreticulin-conjugated Sepharose 4B beads with commercially available azurocidin or myeloblastin to allow them to bind to calreticulin. The beads were then washed well

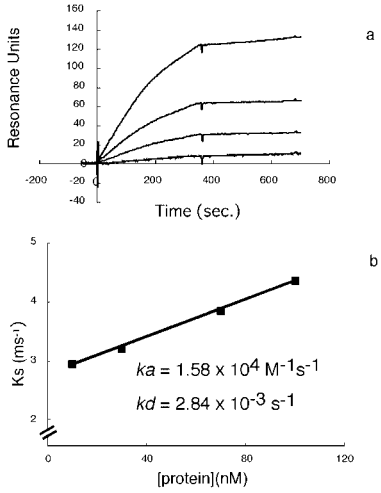

Fig. 4. **Analysis of the binding between azurocidin and calreticulin.** The binding between azurocidin and calreticulin was analyzed using surface plasmon resonance. (a) Overlay plot of sensorgrams depicting the interaction of azurocidin with immobilized calreticulin at 25°C. The concentrations of azurocidin, from bottom to top, were 19, 38, 75, and 150 nM. (b) Scatchard plot of the sensorgram data.

and heated at 75°C for 15 min in electrophoresis buffer to elute the bound protein. We found that azurocidin bound to calreticulin-bound beads, and that the bound azurocidin was not eluted from the beads by simple washing, but by heating at 75°C for 15 min in electrophoresis buffer. Azurocidin did not bind to control Sepharose 4B beads without calreticulin (Fig. 2). When the same experiment was performed with myeloblastin, the myeloblastin was recovered in the unbound or wash fraction, *i.e.*, no appreciable binding of myeloblastin to calreticulin was detected (data not shown). Thus, we conclude that only azurocidin exhibits affinity for calreticulin.

We further investigated the binding between azurocidin and calreticulin, and found that azurocidin is able to bind to both the P-domain and C-domain of calreticulin (Fig. 3, a and b). In this experiment, we employed α-chymotrypsinogen as a negative control (Fig. 3b). We also prepared the recombinant N-domain of calreticulin, but this peptide was insoluble and could not be used for the binding experiment.

All calreticulin and azurocidin binding experiments were conducted using recombinant rabbit calreticulin and its structural domains. However, as we were able to obtain recombinant human calreticulin, we confirmed the binding of azurocidin and human calreticulin using a surface plasmon resonance approach. As shown in Fig. 4a, azurocidin bound to immobilized calreticulin in a dose-dependent manner, with the linear fitting model indicating association and dissociation constants of $1.58 \times 10^4\ M^{-1}\ s^{-1}$ and $2.84 \times 10^{-3}\ s^{-1}$, respectively. The K_d value of the interaction was 1.80×10^{-7} [M] (Fig. 4b), whichh is in the range of K_d values obtained for interactions of other molecular chaperones and their substrates (*22*).

Activation of Peripheral Monocytes by Azurocidin through Cell Surface Calreticulin—As reported previously, human neutrophils are activated by an antibacterial peptide, L5, through cell surface calreticulin to pro-

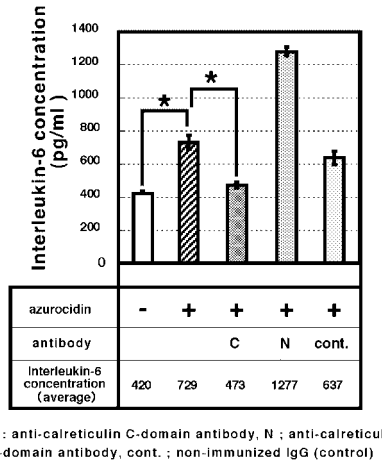

Fig. 5. **Inhibition of azurocidin-induced IL-6 production by anti-calreticulin antibody.** Freshly prepared peripheral monocytes were treated with 10 μg/ml of azurocidin, or azurocidin and 50 μg/ml of antibody (anti-calreticulin C-domain peptide antibody, anti-calreticulin N-domain peptide antibody, or non-immunized IgG) for 24 h. Then the amount of IL-6 in the culture medium was measured by the sandwich-ELISA method. The averages of duplicate measurements are shown with the deviation. $*p < 0.05$.

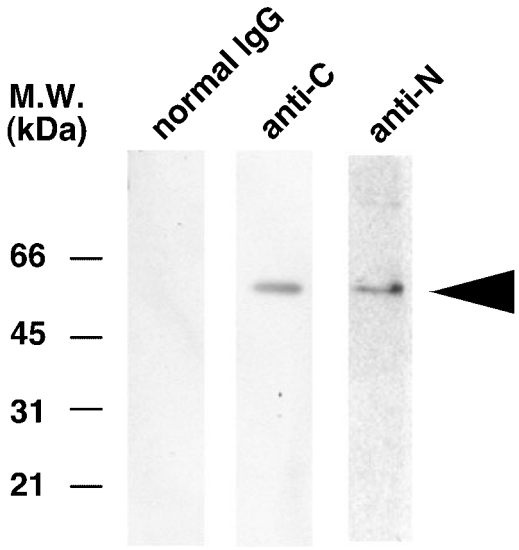

Fig. 6. **Specificity of the antibodies.** Immunoblotting of human monocyte lysate was performed using an anti-calreticulin N-domain and C-domain peptide antibodies. Each lane contained 50 μg of protein. Normal IgG, non-immunized rabbit IgG; anti-C, anti calreticulin C-domain peptide antibody; anti-N, anti calreticulin N-domain peptide antibody. The arrow indicates the position of authentic calreticulin.

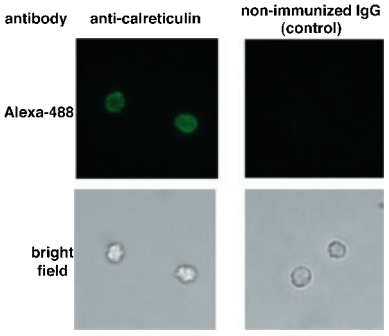

Fig. 7. **Immunofluorescence study of monocyte-surface calreticulin.** Human monocytes were treated with the anti-calreticulin C-domain peptide antibody or non-immunized IgG (control), followed by Alexa-488-conjugated mouse anti-rabbit antibody to visualize calreticulin.

duce O_2^- (*12*). We examined whether or not azurocidin activates neutrophils such as L5, as azurocidin was found to bind to calreticulin. However, no appreciable O_2^- production was detected when neutrophils were incubated in the presence of azurocidin (data not shown).

Azurocidin is known to induce human monocytes to produce inflammatory cytokines such as IL-6 (*23–25*). To determine whether or not calreticulin is involved in this process, we investigated the activation of monocytes through cell surface calreticulin. As summarized in Fig. 5, the production of IL-6 by monocytes was enhanced about 1.7-fold in the presence of 10 μg/ml of azurocidin. Treatment with 1 μg/ml of azurocidin induced no significant IL-6 generation (data not shown). The background level of IL-6 production was 420 pg/ml, which is in the normal range, but the level increased to 729 pg/ml in the presence of azurocidin, confirming the results of Rasmussen *et al.* (*24*). The enhancement of IL-6 production by azurocidin was inhibited (83% inhibition) by an anti-calreticulin C-domain peptide antibody, but not so strongly (32% inhibition) by control IgG. We also examined the effect of the anti-C domain peptide antibody in the absence of azurocidin, but the background level of IL-6 production was not affected by the antibody. Interestingly, no appreciable inhibition was detected with anti-calreticulin N-domain peptide antibody; instead the anti N-domain antibody rather enhanced the production of IL-6 to about 2.9-fold over the background level, although the reason for this is unknown. To examine the specificities of the antibodies, we performed immunoblotting using U937 cell lysates. As shown in Fig. 6, a single band of calreticulin was detected with both anti-calreticulin N-domain and C-domain peptide antibodies, indicating that these antibodies are specific for calreticulin.

These facts suggest that calreticulin is also located on the surface of monocytes and participates in the signal transduction process, as has been observed for neutrophils. To confirm the cell surface localization of calreticulin, we performed an indirect immunofluorescence study using non-permeable human monocytes. Contrary to non-immunized rabbit IgG, clear fluorescence was detected when the cells were treated with the anti-calreticulin C-domain peptide antibody (Fig. 7), indicating that calreticulin is present on the surface of monocytes.

State of Cell Surface Calreticulin—Judging from its amino acid sequence, calreticulin does not seem to contain a transmembrane domain. However, our results suggest that it has to be anchored to the cell surface. In order to know the state of calreticulin on the monocyte surface, we treated monocytes with 2 M KCl or 66.7 mM PtdIns-specific phospholipase C. Then, the cells were subjected to indirect immunofluorescence with anti-calreticulin C-domain peptide antibody to determine the amount of cal-

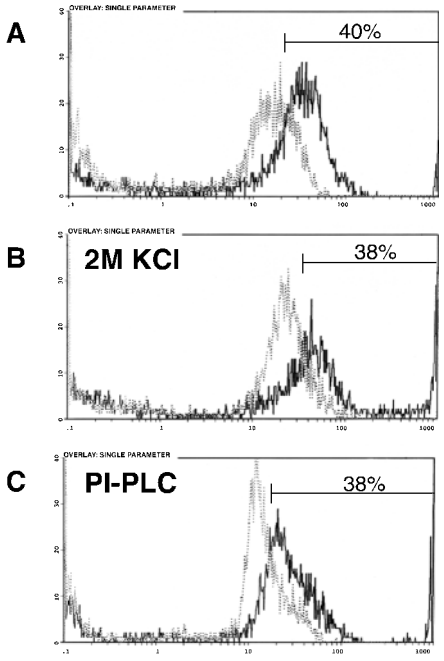

Fig. 8. **Calreticulin on the human monocyte surface.** Human monocytes were suspended in 10 mM Hepes/NaOH (pH 7.4), 150 mM NaCl, and 1 mM EGTA, and treated with 2 M KCl or 66.7 mM PtdIns-specific phospholipase C for 60 min at 37°C. Then the cells were subjected to indirect immunofluorescence using an anti-calreticulin C-domain peptide antibody (bold lines) or non-immunized rabbit IgG (dotted lines). (A) non-treated cells (control), (B) cells treated with 2 M KCl, (C) cells treated with 66.7mM PtdIns-specific phospholipase C.

reticulin remaining on the cell surface. Data were collected using EPICS ELITE and analyzed by Immuno-4 (Beckman Coulter, Brea, California). As shown in Fig. 8, no appreciable loss of cell surface calreticulin was detected even when the monocytes were treated with high salt or PtdIns-specific phospholipase C. These results suggest that the anchoring mechanism of calreticulin to the cell surface is not simple.

DISCUSSION

During a study to Identify a neutrophil membrane protein that mediates signal transduction by the L5 peptide *via* cell surface calreticulin, we incidentally found that azurocidin exhibits affinity for calreticulin. Our present study suggests that calreticulin on the surface of monocytes is involved in IL-6 production induced by azurocidin, conceivably as a receptor for azurocidin. Various cells have been reported to have cell surface calreticulin (*2*, *7–9*, *26–28*), although its function *in situ* is not clear.

Several points require discussion. In this study, we initially identified both azurocidin and myeloblastin as calreticulin-binding proteins, but it turned out that only azurocidin exhibits affinity for calreticulin. Possibly, myeloblastin forms a complex with azurocidin that has affinity for calreticulin, although myeloblastin itself has no affinity for calreticulin.

In a previous paper, we demonstrated that the binding of L5 to calreticulin on the cell surface of neutrophils triggers O_2^- production. However, nothing happened when the neutrophils were treated with azurocidin, although azurocidin was assumed to bind to cell surface calreticulin. In contrast to neutrophils, monocytes were induced by azurocidin to produce IL-6. These facts suggest that calreticulin is a common receptor for multiple ligands, and different signals are transmitted to cells depending upon the ligand bound, resulting in the activation of a specific signal transduction pathway for the ligand. Possibly, the conformation of calreticulin in monocytes is different from that in U937 cells, and calreticulin in the former cells forms a fertile complex with azurocidin, while in the latter cells it forms complexes with L5, with different signals being transmitted into the cells.

We found that azurocidin can bind to both the P-domain and C-domain of calreticulin. It is known that calreticulin has lectin activity carried by the P-domain (*29*). As azurocidin is a glycosylated protein, it is possible that azurocidin binds to the P-domain *via* its carbohydrate chain. The binding between azurocidin and the C-domain may be due to an ionic interaction, as the former is a basic protein and the latter is a very acidic region of calreticulin. Although azurocidin was found to bind to these domains *in vitro*, the binding does not necessarily reflect the fertile binding of azurocidin and intact calreticulin, as there remains the possibility that azurocidin recognizes the tertiary structure of intact calreticulin on the cell surface.

It is noteworthy that the anti-calreticulin C-domain peptide antibody inhibited the azurocidin-induced IL-6 production while the anti-calreticulin N-domain peptide antibody rather stimulated it. A similar antibody effect has been detected when O_2^- production was induced by the L5 peptide in U937 cells (*30*), but the effects of the antibody were opposite, that is, the anti-calreticulin N-domain peptide antibody inhibited O_2^- production, whereas the anti-calreticulin C-domain peptide antibody enhanced it. However, when peripheral neutrophils were employed, both antibodies inhibited O_2^- production induced by L5 in the same way (*12*).

It is known that an antibody against a certain receptor sometimes mimic the ligand for that receptor, resulting in the induction of signal transduction. A similar situation may occur in the case of calreticulin. When the anti-calreticulin N-domain peptide antibody binds to residues 54–73 (N-domain peptide) of monocyte surface calreticulin, IL-6 production is enhanced. Whereas, when the anti-calreticulin C-domain peptide antibody binds to residues 399–417 (C-domain peptide) of U937 surface calreticulin, O_2^- production is enhanced. However, the molecular mechanisms of signal transduction mediated by cell surface calreticulin remain to be elucidated.

Our results suggest that cell surface calreticulin is not a glycosylated PtdIns-anchored protein. Moreover, as it does not seem to be removed from the cell surface simply by treating the cells with 2 M KCl, the interaction between calreticulin and the cell surface is very firm.

It is known that azurocidin exhibits chemotactic activity toward monocytes (*23–25*, *31–35*). Recently, azurocidin was shown to induce Ca^{2+}-dependent cytoskeletal rearrangement and intercellular gap formation in endothelial-cell monolayers, and to increase macromolecular efflux from microvessels (*36*). These responses are

assumed to be receptor-mediated ones and cell surface calreticulin is likely to be involved in these cellular responses induced by azurocidin.

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