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Generation of monoclonal antibodies against a soluble form of lectin-like oxidized low-density lipoprotein receptor-1 and development of a sensitive chemiluminescent enzyme immunoassay

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ABSTRACT

Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), expressed prominently in atherosclerotic lesions, is cleaved and released as a soluble LOX-1 (sLOX-1), which is a specific biomarker to diagnose acute coronary syndrome (ACS) at an early stage. Although sLOX-1 levels in patient's blood were successfully measured with our previously established enzyme-linked immunosorbent assay (ELISA), the assay was not sensitive enough to detect normal serum levels of sLOX-1 in healthy human subjects. We therefore developed sensitive and specific monoclonal antibodies (mAbs) against sLOX-1 in order to establish a more sensitive immunoassay. Mice were immunized with recombinant human LOX-1 extracellular domain. mAbs were subsequently generated by standard myeloma cell fusion techniques with a novel screening method using time-resolved fluorescence immunoassay. Using two anti-human sLOX-1 mAbs and alkaline phosphatase as a label, a sandwich chemiluminescent enzyme immunoassay (CLEIA) was developed. In total, nine mAbs were obtained. The dissociation constant (K_d) values of these mAbs for sLOX-1 were 0.12–1.32 nM. Characteristics of these mAbs were estimated and the best combination for CLEIA was selected. The newly established CLEIA could determine sLOX-1 levels as low as 8 pg/mL, and thus, was sensitive enough to measure serum sLOX-1 levels in normal human subjects and to evaluate subtle differences. Values for sLOX-1 measured by monoclonal CLEIA and polyclonal ELISA were highly correlated ($r^2 = 0.7594$, $p < 0.0001$). Area under the curve values of the receiver-operating characteristic curves in detecting ACS were 0.948 and 0.978 for monoclonal CLEIA and polyclonal ELISA, respectively. Thus, a more sensitive sLOX-1 CLEIA was established using newly developed mAbs against sLOX-1. In addition to its advantage in early diagnosis of ACS, this assay may also be useful in predicting cardiovascular disease risk in disease-free subjects.

1. Introduction

It is widely recognized that acute coronary syndrome (ACS) is caused by rupturing of lipid-rich atheromatous plaques followed by thrombus formation [1,2]. Biomarkers for plaque instability or rupture would allow prediction or accurate diagnosis of ACS at an early stage. Currently, the MB fraction of creatine kinase (CK-MB), troponin T, and heart-type fatty acid-binding protein (H-FABP) are used as serum biomarkers of ACS [3–5]. However, these markers are not markedly elevated during the early stage of ACS, before ischemic myocardial damage becomes apparent. In addition, if ischemic myocardial damage remains minimal, these biomarkers may not be significantly elevated even several hours after the onset of ACS. Several other serum biomarkers, including high-sensitivity C-reactive protein (hs-CRP), were reported to be associated with ACS [6]. However, none of these markers have been established as clinical tests for ACS because they do not directly or specifically indicate plaque instability or plaque rupture before myocardial damage becomes apparent.

For this purpose, we focused on lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) [7]. In human atherosclerotic lesions, LOX-1 is prominently expressed by intimal smooth muscle cells and macrophages in advanced atherosclerotic plaques and by vascular endothelial cells covering early atherosclerotic lesions

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[8,9]. In contrast, LOX-1 expression is undetectable in normal aortas without visible atherosclerosis [8,9]. LOX-1 expressed on the cell surface can undergo proteolysis at its membrane-proximal extracellular domain (ECD) and be released as soluble LOX-1 (sLOX-1) [10]. Therefore, we previously developed a sandwich enzyme-linked immunosorbent assay (ELISA) for determination of sLOX-1 concentration in human sera, using rabbit polyclonal antibodies and colorimetric detection [11]. Using this technique, we measured serum samples from 427 patients undergoing coronary angiography and from 94 patients with other acute and chronic noncardiovascular diseases, and found that serum sLOX-1 levels are markedly higher in ACS, but not in noncardiovascular acute inflammatory diseases. In addition, receiver-operating characteristic (ROC) curves of sLOX-1 showed higher sensitivity and specificity for the diagnosis of ACS than hs-CRP [12].

Although sLOX-1 in ACS patient sera was successfully measured by our sandwich ELISA, this assay was not sensitive enough to determine the normal serum sLOX-1 levels in healthy human subjects as they are below its lower detection limit. In this study, therefore, sensitive and specific monoclonal antibodies (mAbs) against human sLOX-1 were generated and a more sensitive sandwich chemiluminescent enzyme immunoassay (CLEIA) was developed using a combination of two different mAbs.

2. Materials and methods

2.1. Reagents

All chemicals purchased were of analytical grade unless otherwise specified.

2.2. Preparation of LOX-1 ECD

Recombinant human LOX-1 ECD was produced in *Escherichia coli* by transforming a cDNA fragment corresponding to the ECD of human LOX-1 (GenBank accession no. AB010710), which had been subcloned into a pQE vector (Qiagen, Valencia, CA, USA) [13]. LOX-1 ECD was extracted from *E. coli*, solubilized with 8 M urea, and purified with an Ni-NTA agarose column (Invitrogen, Carlsbad, CA, USA). The eluate with 250 mM imidazole was subjected to stepwise dialysis against 4, 2, 1, and 0 M urea in phosphate buffer (pH 9.0; 0.05 M). The supernatant of the dialysate was used as an assay standard [11] after determination of its concentration by amino acid analysis with a Hitachi L-8800 analyzer (Hitachi High-Technologies, Tokyo, Japan). The amino acid sequence of LOX-1 ECD was obtained as Met Arg Gly Ser His His His His His His Gly – Ser85–Gln273 (of LOX-1) from its cDNA sequence analysis.

2.3. Preparation of human sLOX-1 from CHO cell culture

CHO-K1 cells stably expressing human LOX-1 (LOX-1–CHO) were prepared and cultured as described previously [13]. A small amount of sLOX-1 in the conditioned medium of LOX-1–CHO was concentrated and purified as described previously in bovine sLOX-1 [10].

2.4. Immunization of mice

LOX-1 ECD was also used as an antigen to immunize mice. A solution containing the precipitate of LOX-1 ECD was emulsified with Freund's complete adjuvant and injected intraperitoneally (0.1 mg/0.1 mL) into female mice (A/J Jms Slc; Japan SLC, Hamamatsu, Japan). Mice were immunized a total of four times at 3 week intervals. Small samples of blood were collected from the mice 10 days after each immunization and antibody titers were monitored with a time-resolved fluorescence immunoassay (TR-FIA) as described below.

2.5. Generation of mAb-secreting hybridoma clones

Stimulated mouse spleen cells were harvested after the fourth immunization and mixed with the cells of P3-X63-Ag8-U1 mouse myeloma cell line at a ratio of 5:1. Cell fusion was performed by the polyethylene glycol method. After hypoxanthine, aminopterin, and thymidine (HAT) selection, hybridomas were screened using competitive TR-FIA as described below. Hybridomas that secreted abundant anti-sLOX-1 antibodies were cloned by limiting dilution.

2.6. Production and purification of mAbs

Anti-sLOX-1 antibody-producing clones were cultured in 50 mL flasks or injected intraperitoneally into peristan-pretreated mice. The hybridoma cell culture supernatant or ascites of the hybridoma-injected mice was collected and purified using a protein A affinity column (MAPS-II Kit; Bio-Rad, Hercules, CA, USA).

2.7. Characterization of mAb

2.7.1. Titers of antibodies

Titers of mAbs were determined using TR-FIA. Anti-mouse IgG antibodies (goat IgG, 1 μg/100 μL) in an immobilization buffer [Tris–HCl buffer (pH 7.8; 0.05 M) containing 0.5 g/L sodium azide] were added to microplates (MaxiSorp FluoroNunc; Nalge Nunc, Rochester, NY, USA) and incubated overnight at 4 °C to immobilize on the surface of microplates. After two washes with a blocking buffer [Tris–HCl buffer (pH 7.8; 0.025 M) containing 0.25 g/L sodium azide, 20 g/L Block Ace (Dainippon Sumitomo Pharma, Osaka, Japan), and 100 g/L sucrose], the plates were incubated with the blocking buffer for 2 h at room temperature, dried under vacuum at room temperature, and then stored at 4 °C until use.

LOX-1 ECD was subjected to biotinylation using Sulfo-NHS-LC-Biotin (Thermo, Rockford, IL, USA). The biotin-labeled antigen was mixed with europium-labeled streptavidin (DELFIA Eu-labeled Streptavidin; PerkinElmer, Fremont, CA, USA) to prepare a europium-labeled antigen.

The test antiserum or culture supernatant of hybridoma was diluted serially with an assay buffer [Tris–HCl buffer (pH 7.4; 0.05 M) containing 0.5 g/L sodium azide, 5 g/L bovine serum albumin (BSA), 9.8 mg/L diethylenetriaminepentaacetic acid, 0.1 g/L Tween 80, and 9 g/L sodium chloride] to determine the titer of the antibody. The europium-labeled antigen (100 μL) and the test antibody (50 μL) were added to anti-mouse IgG antibody-immobilized microplates after two washes with a TR-FIA washing solution (saline containing 0.1 g/L Tween 20 and 0.5 g/L sodium azide). The microplates were incubated for 16 h at 4 °C, then washed three times with the TR-FIA washing solution, and then 150 μL of an enhancement solution (1.39 g potassium hydrogen phthalate, 6.0 g acetic acid, 19.3 mg tri-n-octylphosphine oxide, 4.59 mg 2-naphthoyltrifluoroacetone, and 1.0 g Triton X-100 in 1 L distilled water) was added to measure time-resolved fluorescence using a multilabel counter for microplate (Wallac 1420 Arvo$_{sx}$, PerkinElmer). Titers of the antibody were estimated as the dilution of sample solution giving a time-resolved fluorescence intensity of 100,000 counts/s (cps).

2.7.2. Affinity of mAb

Affinities of mAbs were determined by competitive TR-FIA. The europium-labeled antigen solution (50 μL), the culture supernatant of the hybridoma (50 μL) and LOX-1 ECD standard solution (50 μL) or sLOX-1 solution (50 μL) from the culture supernatant of LOX-1–CHO were added to the anti-mouse IgG antibody-immobilized

microplates. Then the microplates were incubated for 16 h and the time-resolved fluorescence intensity was measured as described above. An inhibition curve was obtained using different concentrations of LOX-1 ECD or sLOX-1 derived from the culture supernatant of LOX-1–CHO. Dissociation constants were calculated by Scatchard analysis of the inhibition curves for the mAbs [14].

Immunoglobulin class and subclass were determined using mouse immunoglobulin isotyping ELISA kit (BD Biosciences, San Jose, CA, USA).

2.7.3. Epitope mapping

Epitopes recognized by the mAbs were determined by scanning 63 peptides (15-meric peptides in which sequences of 12 amino acids overlapped) derived from LOX-1 ECD (residues 85–273) using an epitope mapping method [15]. The assay was performed by JPT Peptide Technologies (Berlin, Germany) using their protocols [16].

2.8. Development of CLEIA

2.8.1. Preparation of enzyme-labeled antibody

The mAbs were digested by pepsin (Sigma, St. Louis, MO, USA) and purified to $F(ab')_2$, which was further reduced to Fab′ by 10 mM 2-mercaptoethylamine in buffer A [phosphate buffer (pH 6.0; 0.1 M) containing 5 mM EDTA] by the standard method [17]. Alkaline phosphatase (ALP; 2.0 mg or 14.2 nmol from calf intestine; Kikkoman, Chiba, Japan) in 0.475 mL of a buffer [Tris–HCl buffer (pH 7.0; 0.1 M) containing 1 mM $MgCl_2$ and 0.1 mM $ZnCl_2$] was mixed with 31 μg (71 nmol) of *N*-(8-maleimidocapryloxy) sulfosuccinimide (Sulfo-HMCS; Dojindo, Kumamoto, Japan) in 0.05 mL water for 1.5 h on ice. HMCS-activated ALP was purified using a gel filtration column (PD-10; GE Healthcare, Chalfont St. Giles, UK) and concentrated using an ultracentrifugal concentrator (Centricon YM-30; Millipore, Billerica, MA, USA). The HMCS-activated ALP solution (0.96 mg in 0.192 mL) was added to 0.441 mg of the Fab′ in 0.15 mL of buffer A and mixed for 16 h at 4 °C. The reaction mixture was purified by gel-permeation HPLC [column, TSK gel $G2000SW_{XL}$, id 7.8 mm × 300 mm (TOSOH, Tokyo, Japan); mobile phase, Tris–HCl buffer (pH 6.8; 0.1 M) containing 1 mM $MgCl_2$ and 0.1 mM $ZnCl_2$; flow rate, 0.5 mL/min; detection, UV 280 nm] to obtain ALP-labeled mAb.

2.8.2. Preparation of antibody-immobilized plates

The anti-sLOX-1 mAb (IgG; 1.5 μg in 150 μL immobilization buffer) was added to the microplate wells (MaxiSorp FluoroNunc) and incubated overnight at 4 °C for them to immobilize. After two washes with CLEIA washing solution (saline containing 0.1 g/L Tween 20 and 0.2 g/L sodium azide), the microplates were incubated with the blocking buffer for 2 h at room temperature, dried under vacuum at room temperature, and stored at 4 °C until use. The microplates were washed with the CLEIA washing solution immediately before use.

2.8.3. CLEIA procedure

Standard sLOX-1 solutions (8–25,000 pg/mL LOX-1 ECD) in CLEIA buffer [Tris-buffered saline (pH 7.4; 0.05 M) containing 10 g/L BSA, 0.1 g/L Tween 80, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 10 mg/L mouse γ-globulin, 1000 KIU/mL aprotinin, and 0.5 g/L sodium azide] or serum samples from human subjects (10 μL) were added to the anti-sLOX-1 mAb-immobilized microplate wells in duplicates, containing 100 μL of the CLEIA buffer (serum sample without pretreatment was diluted 1:11). The microplate was sealed after gentle mixing and incubated for 4 h at room temperature (first incubation), which was followed by washing three times with 350 μL CLEIA washing solution. Then, the ALP-labeled mAb solution (100 μL; approximately 94.5 ng/mL in the CLEIA buffer) was added to the microplate wells, and the wells were incubated for 1 h at room temperature (second incubation). This was followed by washing four times and addition of substrate solution (100 μL) containing APS-5 (Lumigen, Southfield, MI, USA). The chemiluminescence (CL) intensity (in cps) of each well was measured using a plate reader (Wallac 1420 $Arvo_{sx}$).

Table 1
Characteristics of anti-sLOX-1 monoclonal antibody clones.

Clone	Affinity to LOX-1 ECD	Affinity to sLOX-1 (CHO)[b]	Growth	Sub class
6B11	0.34 nM[a]	++	++	IgG_1
1G2	+++	+++	++	IgG_1
2E4	+++	+++	++	IgG_1
2E5	++	++	++	IgG_1
2G11	0.16 nM[a]	++	++	IgG_1
3E12	1.32 nM[a]	++	++	IgG_1
7G1	0.12 nM[a]	++	++	IgG_1
1A7	0.51 nM[a]	++	++	IgG_1
1B8	++	++	++	IgG_{2b}

[a] Dissociation constant (K_d).
[b] sLOX-1 was derived from the cell culture supernatant of LOX-1–CHO.

3. Results and discussion

3.1. Assay standard and immunogen

A small amount of sLOX-1 from LOX-1–CHO was obtained and used for sequence analysis and characterization of mAb. The sequence of sLOX-1 was defined as a mixture of residues 88–273 and 92–273. However, appropriate amounts of natural sLOX-1 for use as an assay standard and immunogen could not be obtained after various trials. We decided to use LOX-1 ECD (residues 85–273) as the assay standard and immunogen. We overcame some of the differences between natural sLOX-1 and LOX-1 ECD by taking advantage of the characteristics of immunological methods described below.

3.2. Production of mAbs

In our initial attempt, we produced mAbs against LOX-1 ECD by a standard method; however, the antibodies we obtained reacted only with precipitated (denatured) LOX-1 ECD and not with sLOX-1. We then sought to obtain mAbs that could react with sLOX-1 by immunizing A/J mice in order to obtain higher antibody titers using a novel screening method called competitive TR-FIA.

Consequently, using LOX-1 ECD as an antigen, we obtained nine mAbs (6B11, 1G2, 2E4, 2E5, 2G11, 3E12, 7G1, 1A7, and 1B8) which bound not only to LOX-1 ECD but also to sLOX-1 derived from the cell culture supernatant of LOX-1–CHO. The characteristics of these anti-sLOX-1 mAbs are summarized in Table 1. Affinities of the mAbs to LOX-1 ECD were estimated by Scatchard analysis, although some of them could not be calculated because of absence of linear relationships in their Scatchard plots. Most of the dissociation constants (K_d) were less than 1 nM, indicating that the antigen-binding affinities of these antibodies were high enough for use in sensitive immunoassays.

3.3. Selection of mAbs for sensitive sandwich immunoassay

Seventy-two combinations using eight biotin-labeled mAbs (1B8 could not be labeled) and nine immobilized antibodies were investigated to select suitable mAb pairs for the ability to detect sLOX-1 in a two-site sandwich TR-FIA system. As shown in Table 2, the combination of 6B11 and 1A7 showed the best response and we selected them for the two-site sandwich immunoassay.

Table 2
Combination of monoclonal antibodies for sLOX-1 sandwich immunoassay.

No.	Immobilized antibodies								
	6B11	1A7	3E12	1G2	2G11	2E4	7G1	2E5	1B8
Biotinylated antibodies									
6B11	–	++	–	–	–	–	–	–	–
1A7	++	–	–	–	–	–	–	–	–
3E12	+	–	–	–	–	–	–	–	–
1G2	+	–	–	–	–	–	–	+	–
2G11	+	–	–	–	–	–	–	+	–
2E4	–	–	–	–	–	–	–	–	–
7G1	–	–	–	–	–	–	–	–	–
2E5	–	–	–	–	–	–	–	–	–
1B8	None	None	None	None	None	None	None	None	None

LOX-1 ECD was used as the analyte (antigen).

3.4. Antibody-binding site

The C-type lectin-like domain (CTLD, residues 143–273) of LOX-1 forms a disulfide-linked homodimer [18,19] with Cys140, and therefore, natural sLOX-1 and LOX-1 ECD should exist as a homodimer. Generally, a labeled antibody and an immobilized antibody derived from the same mAb clone would bind to the two structurally identical sites of one homodimer molecule if the mAb can bind to the monomer. However, LOX-1 ECD was not sandwiched by any of the identical mAb pairs, as shown in Table 2. Moreover, neither 6B11 nor 1A7 was able to bind to the denatured LOX-1 ECD in western blotting (data not shown). These results suggest that 6B11 and 1A7 may bind to sites that appear on the natural homodimeric form of sLOX-1.

From the results of epitope mapping, the mAbs 6B11 and 1A7 had almost the same binding profiles at residues 147–158, 195–203, 201–215, and 222–236. Among these residues, 195–203 and 201–205, which are positioned at the engaging surface of the sLOX-1 homodimer, showed stronger binding to both the mAbs. Thus, we presumed that 6B11 and 1A7 may bind to the two steric structures around the engaging surface. Although the precise binding positions have not yet been fully clarified, CLEIA using 6B11 and 1A7 appears to be sufficiently specific because it measures only the homodimeric structure of sLOX-1.

3.5. Development of CLEIA

3.5.1. Assay conditions

We selected 1A7 as the immobilized antibody on microplate wells and 6B11 as the enzyme-labeled antibody for detection. Binding to the immobilized 1A7 IgG (first incubation) was equilibrated for approximately 2 h at room temperature without shaking (data not shown). Therefore, the first incubation time was fixed at 4 h. Binding of ALP-labeled 6B11 Fab′ (second incubation) was equilibrated within 1 h at room temperature without shaking (data not shown). Consequently, 1 h was selected as the standard second incubation time. The incubation times for CLEIA using these mAbs were shorter than those of ELISA (18 h) using polyclonal antibodies [11], and the assay results could be obtained within a day.

3.5.2. CLEIA standard curve, recovery, and precision

Our previous ELISA using polyclonal antibodies was not sensitive enough to be able to determine the circulating sLOX-1 levels in normal healthy human subjects [11,12]. In this study, we developed a more sensitive CLEIA using newly developed anti-sLOX-1 mAbs, ALP as an enzyme label, and APS-5 as a chemiluminescent substrate. CLEIA standard curve for LOX-1 ECD was linear on log (sLOX-1 concentration)-log (chemiliminescence intensity after subtraction of the blank value, 1163 cps) scale, ranging from 8 to 25,000 pg/mL. Precisions were 14.4, 6.3, 5.2, 4.2, 4.6, and 3.3% of coefficient of variation (CV, $n = 10$) at 8, 40, 200, 1000, 5000, and 25,000 pg/mL of standard concentrations, respectively.

Table 3
Intra- and inter-assay precision of monoclonal chemiluminsecent enzyme immunoassay.

STD[a] Added	Measured (pg/mL) Mean ± S.D.[c]	CV[b] (%)	Bias (%)
Intra-assay ($n = 5$)			
0	200 ± 13	6.5	–
100	297 ± 15	5.1	−3.0
1000	1130 ± 50	4.4	−7.0
5000	4880 ± 310	6.4	−6.4
Inter-assay[d] ($n = 15$)			
0	198 ± 12	6.1	–
100	298 ± 12	4.0	0.0
1000	1160 ± 50	4.3	−3.8
5000	4880 ± 230	4.7	−6.4

[a] LOX-1 ECD was used as the assay standard (STD) and added to normal human serum.
[b] Coefficient of variation (CV).
[c] Standard deviation (S.D.).
[d] Overall mean, S.D., and CV for three batches were calculated.

Next, we estimated intra- and inter-assay precision using sera spiked with LOX-1 ECD. As shown in Table 3, CV values (intra-assay CV 4.4–6.5% and inter-assay CV 4.0–6.1%) were good enough. Effects of serum on sLOX-1 measurement were negligible, although standard LOX-1 ECD solutions were prepared without a serum component. We were able to measure 8–25,000 pg/mL of sLOX-1 from the standard curve. Thus, this new CLEIA was 125-fold more sensitive than our previous polyclonal antibody ELISA with colorimetric detection (quantification range 1–100 ng/mL) [11]. In addition, samples with concentration higher than 25,000 pg/mL (25, 50, 100 ng/mL of spiked samples) could be measured accurately by CLEIA after several fold dilution with the CLEIA buffer (1/1, 1/4, 1/16, 1/64, 1/256, and 1/1024) (recovery 91–104%).

3.5.3. Specificity

We were unable to obtain sufficient amounts of natural human sLOX-1 to be used as a standard; therefore, we examined the reactivity of the mAbs with a natural form of sLOX-1 that was produced in cell-conditioned medium from human aortic endothelial cells (HAEC; Cascade Biologics, Portland, OR, USA) and activated by TNF-α (R&D Systems, Minneapolis, MN, USA). We successfully detected natural human sLOX-1 by CLEIA, as shown in Fig. 1.

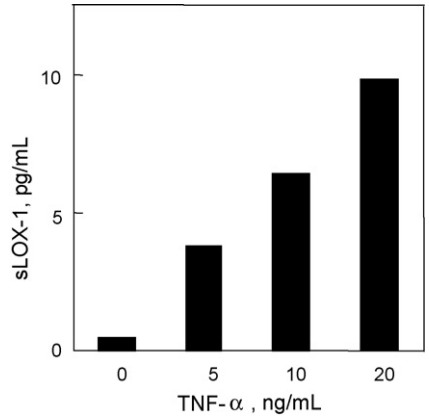

Fig. 1. TNF-α induced sLOX-1 release from human aortic endothelial cells (HAEC). After HAEC were incubated with the indicated concentrations of TNF-α for 24 h, they were further incubated for 24 h in 1% FCS/199 medium. Cell-conditioned media were subjected to the sLOX-1 CLEIA. Values below the assay range (<8 pg/mL) were for reference, calculated by extrapolation.

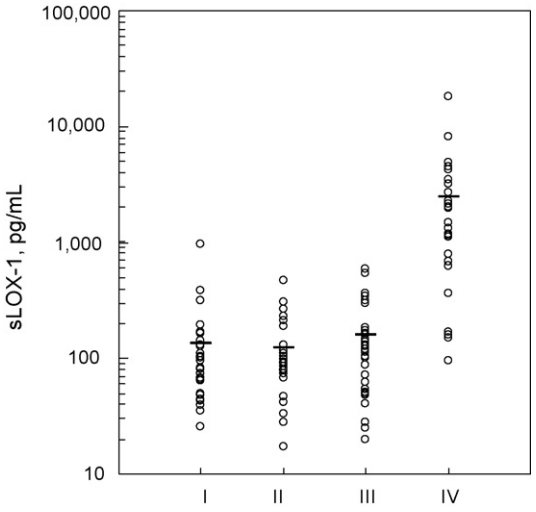

Fig. 2. Diagnosis of ACS by CLEIA. Serum sLOX-1 concentrations in four groups of subjects were measured by CLEIA. Groups I–III consisted of subjects without ACS (total, 90 subjects) and Group IV comprised patients with ACS (30 patients). See Section 3.6 for details of the four groups. Group IV differed significantly from the other groups ANOVA ($p < 0.0001$).

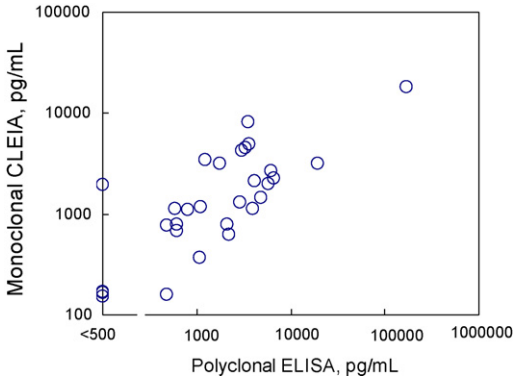

Fig. 3. Correlation of values measured by monoclonal CLEIA and polyclonal ELISA. In ACS (Group IV) samples, the equation relating polyclonal ELISA (x) to monoclonal CLEIA (y) values was $y = 0.09633x + 1751$ ($r^2 = 0.7594$, $p = 0.0000907$, $n = 26$). Values for six points in the polyclonal ELISA that were between the detection limit (0.5 ng/mL) and the limit of quantification (1 ng/mL) were for reference, calculated by extrapolation. Four points below 0.5 pg/mL in the polyclonal ELISA were omitted from the calculations.

The effect of lipoprotein ligands on CLEIA was investigated. Various concentrations of oxidized LDL (up to 500 μg/mL, Biomedical Technologies, Stoughton, MA, USA) or native LDL (as a negative control, up to 1250 μg/mL; Biomedical Technologies) were added to sLOX-1 solutions derived from LOX-1–CHO cell supernatant (600 pg/mL), and then sLOX-1 concentrations were measured by CLEIA after 24 h incubation. All measured values were approximately 600 pg/mL and assay results of CLEIA were not significantly changed by the presence of LOX-1 ligand, oxidized LDL or native LDL (data not shown). These results suggest that the mAb binding site is far from the top (opposite the *N*- and *C*-terminal part) of the CTLD, where oxidized LDL is bound, which agrees with the antibody-binding position described in Section 3.4.

Similarly, various concentrations of bilirubin F, bilirubin C, hemolytic hemoglobin, and chyle (2.3–18.7 mg/dL, 2.6–20.9 mg/dL, 61–484 mg/dL, and 183–1460 FTU, respectively, in final concentration; Interference Check A Plus; Sysmex, Kobe, Japan) were added to normal human serum. Samples were then measured by CLEIA; the assay values were not significantly altered by addition of increasing amounts of these interfering components (data not shown).

It is important to assess cross-reactivities with sLOX-1-like proteins, but we did not find appropriate proteins which had CTLD or identical amino acid sequence. Further studies would be necessary to find any cross-reactive compounds that are present in human serum, especially from patients with diseases including ACS.

3.5.4. Stability of spiked samples

LOX-1 ECD (1000 pg/mL) was added to normal human serum and the samples were measured after five freeze–thaw cycles between −80 °C and room temperature. Remaining immunoreactivity after the five cycles was 97–105%. The assay reagents and the spiked samples were stable under the proposed assay conditions.

3.6. Measurement of serum samples

We measured 26 serum samples collected from normal subjects (11 males and 15 females, age 28–57) after informed consent and successfully determined the sLOX-1 concentrations in all samples by CLEIA. Their mean (±standard deviation) value was 191 (±89) pg/mL. The proposed CLEIA was sensitive enough to measure levels of sLOX-1 in normal human sera.

We then measured 120 serum samples in four patient groups (30 patients each; Group I, intact coronary; Group II, medically controlled stable coronary heart disease [CHD] patients with significant coronary stenosis; Group III, stable CHD patients who required elective percutaneous coronary intervention [PCI] or coronary artery bypass graft surgery [CABG]; and Group IV, ACS) randomly selected from corresponding groups among 427 patients who underwent diagnostic coronary angiography as described previously [12]. As shown in Fig. 2, the current monoclonal CLEIA was able to discrim-

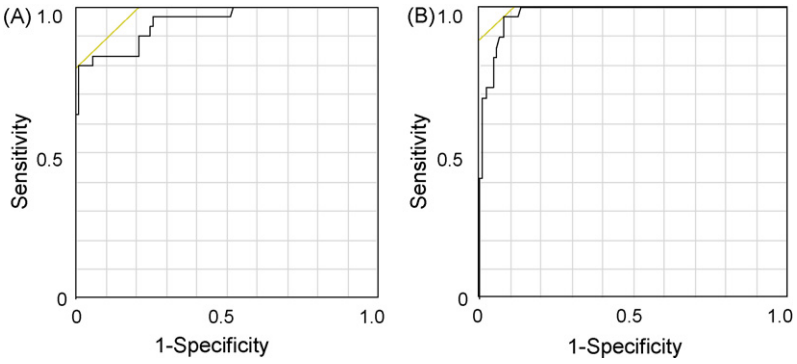

Fig. 4. Comparison of ROC curves of sLOX-1 from monoclonal CLEIA and polyclonal ELISA for the diagnosis of ACS. ROC curves of sLOX-1 measured by monoclonal CLEIA (A) and polyclonal ELISA (B) for the diagnosis of ACS. The true-positive fraction (sensitivity, y axis) was plotted against the false-positive fraction (1–specificity, x axis) by changing cutoff values for the test (sLOX-1). AUC values for monoclonal CLEIA and polyclonal ELISA were 0.948 and 0.978, respectively.

inate ACS patients from others in a similar manner to the previous polyclonal ELISA [12].

There were fairly good correlations between values measured by monoclonal CLEIA and polyclonal ELISA (Fig. 3). The correlation would be improved by better understanding of the stability of sLOX-1 and the differences in assay specificity between polyclonal ELISA and monoclonal CLEIA.

Fig. 4 compares the ROC curves of sLOX-1 values measured by monoclonal CLEIA and the previously reported polyclonal ELISA for the detection of ACS [12]. The area under the curve (AUC) values for monoclonal CLEIA and polyclonal ELISA were 0.948 and 0.978, respectively, indicating sufficient specificity and sensitivity of both the assays in detecting ACS.

We developed a time saving and a very sensitive immunoassay using newly produced mAbs. We believe that this CLEIA could become a useful diagnostic tool for risk prediction or early detection of ACS and other diseases in which sLOX-1 levels are elevated. Furthermore, this assay may prove useful in developing novel drugs to prevent ACS, using sLOX-1 as a surrogate biomarker.

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