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Activation-Dependent Surface Expression of LOX-1 in Human Platelets

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Lectin-like oxidized LDL receptor-1 (LOX-1) was initially identified as an oxidized LDL receptor in aortic endothelial cells. Here we identified LOX-1 mRNA and protein in human platelets in addition to recent findings on the expression in macrophages and smooth muscle cells. The presence of LOX-1 was further confirmed in the megakaryocytic cell lines. Flow cytometric analyses revealed that LOX-1 was exposed on the surface of platelets in an activation-dependent manner. Consistently, the activation-dependent binding of OxLDL to platelets was mostly inhibited by anti-LOX-1 antibody. Immunohistochemistry of the atherosclerotic plaque from a patient with unstable angina pectoris (UAP) revealed accumulation of LOX-1 protein at the site of thrombus. As LOX-1 recognizes and binds activated platelets, exposure of LOX-1 on activated platelets surface might assist thrombosis formation.

Key Words: **LOX-1; oxidized LDL; human platelet.**

Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), a novel oxidized LDL (OxLDL) receptor in vascular endothelial cells, was recently identified in our laboratory. LOX-1 is a type-II membrane protein that belongs to the C-type lectin family (1). LOX-1 has a highest homology to NK-cell receptors, which are implicated in tumor cell recognition and NK-cell activation (2). Consistently, LOX-1 gene is clustered in the NK-cell receptor gene family (3, 4). Functional analysis revealed that LOX-1 binds and uptakes aged/apoptotic cells and activated platelets as well as OxLDL, suggesting potential physiological function of LOX-1 involved in cell-cell interaction (5, 6).

OxLDL has been suggested as an important inducing factor for endothelial dysfunction (7). Actually, OxLDL interacted with LOX-1 elicited many functional changes in endothelial cells, i.e., the expression of cytokines, the increase in cell-adhesion, and the production of reactive oxygen species (8, 9). In addition, some reports suggested that OxLDL might also affect platelet functions (10, 11). OxLDL induces morphological changes, increased adhesiveness, and degranulation in platelets (12–14).

Among the multiple OxLDL receptors identified so far, CD36 is confirmed to be present in platelets and responsible for some of the binding activity (15, 16). Experiments with blocking antibodies for CD36 suggested that CD36 is responsible for a part of the overall binding of OxLDL in human platelets (15, 17, 18). To further characterize the OxLDL binding in platelets, here we investigated the expression of LOX-1 in human platelets.

MATERIALS AND METHODS

Preparation of OxLDL. Human LDL (d = 1.019–1.063) was isolated by sequential ultracentrifugation from fresh plasma collected in 1 mg/mL EDTA, and oxidative modification of LDL was performed with Cu^{2+} *in vitro* as described (1). The degree of oxidation was monitored by measuring the amount of thiobarbituric acid-reactive substances (TBARS). OxLDL contained approximately 10 nmol malondialdehyde equivalent/mg protein (about 0.2 in native LDL). OxLDL was labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI, Molecular Probes) as described (19).

Preparation of washed human platelets. Venous blood was collected from healthy volunteers with 1/10 volume of 3.8% (w/v) sodium citrate. Platelet-rich plasma (PRP) was prepared by centrifug-

Abbreviations used: LDL, low density lipoproteins; OxLDL, oxidized low density lipoproteins; LOX-1, Lectin-like oxidized LDL receptor-1; HEL, human erythroleukemia cells; NK cell, natural killer cells; RT-PCR, reverse transcriptase-polymerase chain reaction; NBCS, newborn calf serum.

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ing the blood at 200*g* for 10 min at room temperature. The upper one-third of the PRP was transferred to a new tube to avoid contamination of nucleated cells. PRP was centrifuged at 1000*g* for 15 min in the presence of 10% ACD buffer (0.8% citric acid, 2.2% sodium citrate, and 2.4% dextrose) to form platelet pellet. The pellet was washed twice with Ca^{2+}-free Tyrode's buffer (134 mmol/L NaCl, 2.7 mmol/L KCl, 1.0 mmol/L $MgCl_2$, 11.9 mmol/L $NaHCO_3$, 0.35 mmol/L NaH_2PO_4, 5.5 mmol/L D-glucose, 3.5 mg/mL BSA and 5.0 mmol/L Hepes, pH 7.4), and resuspended in the appropriate buffer used in each experiment. Resting platelets were prepared with the buffer containing prostaglandin E_1 (5.6 μmol/L). For activation experiments, platelets were prepared without Prostaglandin E_1, and incubated with 0.1 U/mL thrombin in Tyrode's buffer containing 1 mmol/L $CaCl_2$ for 15 min at room temperature. To prevent platelet aggregation, anti-human CD41a monoclonal antibody HIP8 (Pharmingen) at 1 μg/mL was included. After incubation, platelets were fixed by 1% paraformaldehyde for 10 min and then washed and resuspended in PBS containing 0.2% BSA.

Isolation of human monocytes, lymphocytes, and neutrophils. Human peripheral blood mononuclear cells (monocytes and lymphocytes) were isolated from whole blood using Ficoll–Paque (Pharmacia) according to the manufacturer's instructions. To isolate neutrophils, erythrocytes were removed by precipitation with 1.2% Dextran 500 (w/v) for 1 h at room temperature before the preparation with Ficoll–Paque. All cell preparations were washed with PBS and dissolved in appropriate buffer for the following analysis.

Cell culture. Human megakaryocytic cell lines HEL, MEG-01, and MEG-01s were cultured in RPMI 1640 medium (GIBCO) supplemented with 10% FCS. The HEL cells were treated with 1.25% (v/v) of dimethyl sulfoxide (DMSO) for 48 h to induce differentiation into megakaryocytes according to the reference (20).

Isolation of RNA, RT- PCR, and Southern blot analysis. One μg of total RNA isolated from the cells were reverse transcribed into cDNA in 50 μL reaction mixtures as described (1, 21). Five percent of the reaction was amplified with LA-Taq DNA-polymerase (Takara) using a primer pair specific to human LOX-1 cDNA (sense primer: 5′-TGCCTGGGATTAGTAGTGACC-3′; antisense primer: 5′-CCAGTTAAATGAGCCCGAGG-3′). The PCR profile was set at 94°C for 40 s, 56°C 1 min, 68°C 1 min for 35 cycles. Amplified products were blotted onto a nylon membrane (Biodyne), and hybridized with [α-^{32}P]dCTP-labeled human LOX-1cDNA probe under high stringent conditions.

Western and ligand blot analyses. Freshly isolated platelet pellet and other cells were dissolved in lysis buffer containing 10 mmol/L Hepes, 137 mmol/L NaCl, 2.9 mmol/L KCl, 12 mmol/L $NaHCO_3$, 25 mmol/L deoxycholic acid (1%), 7 mmol/L SDS (0.2%) and 1% Nonidet P-40 with protease inhibitors cocktail tablets (Boehringer). The lysates were centrifuged at 16,000*g* at 4°C for 30 min. Equal amounts of protein in the supernatants were subjected to SDS–polyacrylamide (10%) gel electrophoresis, and blotted onto a polyvinylidene difluoride membrane (Immobilon, Millipore). The membrane was probed with a mouse monoclonal antibody against human LOX-1 (#5-2) as described (1, 21). Similarly, a mouse anti-human CD36 antibody (MCA1214, Serotec) identified CD36. To perform the ligand blot analysis, the membrane strips were incubated with or without OxLDL (10 μg/mL) at room temperature for 3 h in binding buffer (50 mM Tris/HCl, 5% BSA, 50 mM NaCl, 1 mM KI, 2 mM $CaCl_2$ pH 8.0) supplemented with 10% newborn calf serum (NBCS, Gibco). The membrane was washed twice with the same buffer. Bound ligand was detected with sheep anti-human apolipoprotein B peroxidase (The binding Site, UK), and then developed by Konica Immunostain kit (Konica, Japan) (1, 22).

Generation of anti-human LOX-1 blocking monoclonal antibody. Chinese hamster ovary (CHO) cells stable expressing human LOX-1 (HLOX-1-CHO) was used as antigen to immunize human immunoglobulin gene transgenic mice. Hybridomas were prepared by standard procedures, and screened by cell-surface immunobinding to HLOX-1CHO cells. The antibodies were further screened for the activity to block the uptake of DiI-labeled OxLDL by human LOX-1 expressing cells. The obtained clone, JTX68, was used for the analysis of LOX-1 activity.

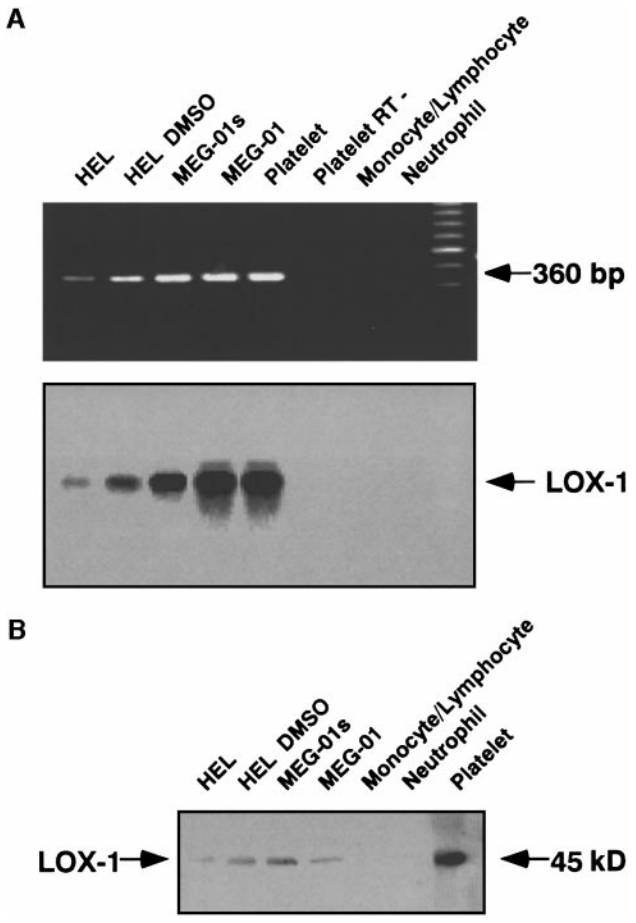

FIG. 1. Molecular characterization of LOX-1 expression in human platelets and megakaryocytes. (A) Identification of LOX-1 mRNA in human platelets by RT-PCR amplification. The specificity of the PCR was confirmed by Southern hybridization (lower panel). RT−: Reverse transcriptase free. (B) Western blot analysis identifies LOX-1 protein in human platelets and megakaryocytic cell lines as a specific band of 45 kDa.

Flow cytometry. The platelet pellet obtained from the above procedure was resuspended and fixed by 1% paraformaldehyde in PBS at pH 7.2 and washed twice with PBS containing 0.2% BSA. Staining with fluorescein isothiocyanate (FITC) conjugated anti-human CD41 antibody (HIP8, Pharmingen) identified the gate of platelet. The platelet suspensions were incubated with mouse anti-human LOX-1 monoclonal antibody (JM-90, humanized IgG) 10 μg/mL at 4°C for 1 h. Cy3-labeled goat anti-human IgG (Amersham) was applied as secondary antibody with 1:400 dilution, and kept at 4°C for 30 min. Similarly, the platelets were incubated with mouse anti-human CD62P antibody (CLB-Thromb/6, Immunotech) followed by FITC-labeled goat anti-mice IgG (Molecular Probes). The stained platelets were washed twice with PBS, fixed in 1% paraformaldehyde, and analyzed by FACScan (Becton–Dickinson).

Binding of OxLDL to human platelets. For platelet binding studies, freshly isolated resting and activated human platelets (5×10^6 cells) were incubated with DiI-labeled OxLDL at final concentration

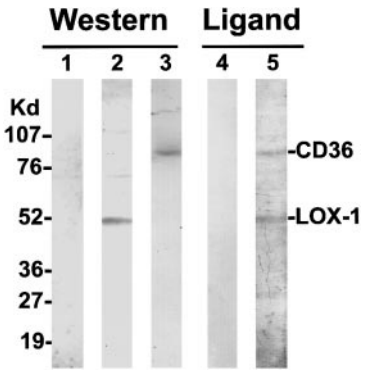

FIG. 2. Ligand blot in combination with Western blot to characterize LOX-1 as a receptor for OxLDL in human platelets. Lanes 1–3 are Western blots: 1, control IgG; 2, anti-LOX-1; 3, anti-CD36. Lanes 4–5 are ligand blots: 4, negative control, without OxLDL; 5, OxLDL. Two major bands were identified in human platelet binding to OxLDL. The band exhibited at about 45 kDa was colocalized with the specific band of LOX-1 in Western blot. The band at 88 kDa was verified as CD36.

of 10 μg/mL in Tyrode's buffer supplemented with 10% NBCS at room temperature for 3 h. After washing with PBS 0.2% BSA twice, the platelets were collected by centrifuging at 3000 rpm for 10 min. Calculation of the bound OxLDL was based on the fluorescence activity of the labeled DiI-OxLDL as described (19). In some experiments, blocking antibodies for CD36 (FA6-152, 2 μg/mL), LOX-1 (JM-90, 10 μg/mL) or isotype-matched normal IgG was added to platelets 30 min before DiI-OxLDL. Nonspecific binding was defined as the binding that was not displaced by a 100-fold excess of unlabeled OxLDL. The specific binding was determined by subtracting the values for nonspecific binding from total binding.

Immunohistochemistry. Human endarterectomy tissue samples were obtained from the coronary artery of a patient with unstable angina pectoris. The samples were snap-frozen and sectioned serially at 6 μm thickness. Immunostaining of the sections with anti-LOX-1 monoclonal antibody (JTX68) and anti-glycoprotein IIb monoclonal antibody (5B12, DAKO) were subjected to a two- or three-step staining procedure with the use of streptavidin-biotin complex with horseradish peroxidase for color detection. Horseradish peroxidase activity was visualized with 3-amino-9-ethylcarbazole, and the sections were counterstained faintly with hematoxylin. To confirm the identity of cell types that stains positive for LOX-1, immunodouble staining with biotinylated anti-LOX-1 antibody and anti-glycoprotein IIb was also performed, according to the procedures previously reported (23). In this staining, alkaline phosphatase was visualized with fast blue BB (blue) and peroxidase with 3-amino-9-ethylcarbazole development (red).

RESULTS

RT-PCR analysis of platelet mRNA showed the presence of LOX-1 mRNA (Fig. 1A). In this condition, LOX-1 mRNA was not detected in circulating human blood monocytes, lymphocytes and neutrophils, further corroborating the integrity of LOX-1 signals from platelets. Consistently, we identified the expression of LOX-1 also in three megakaryocytic cell lines, HEL, MEG-01, and MEG-01s. In HEL cells, the expression of LOX-1 was increased by DMSO which induces the differentiation into megakaryocytes (20, 24). Western blot analysis confirmed the translation of LOX-1 mRNA into protein in platelets and the megakaryotic cell lines (Fig. 1B). LOX-1 protein in human platelets and megakaryotic cell lines was detected as about 45 kDa protein, consistent with that in transfected cells and aortic endothelial cells (1)

Then, we analyzed the binding proteins for OxLDL in human platelets by ligand blotting. As shown in Fig. 2, two major bands were observed. One of the major bands in ligand blotting was co-localized with the specific band of LOX-1 in Western blotting. The other was colocalized with the band of CD36 in Western blotting. This result confirmed that LOX-1, as well as CD36 is the major binding protein for OxLDL in human platelets.

As LOX-1 expression in endothelial cells is quite inducible by inflammatory cytokines, OxLDL, etc., here we examined the manner of regulation of the

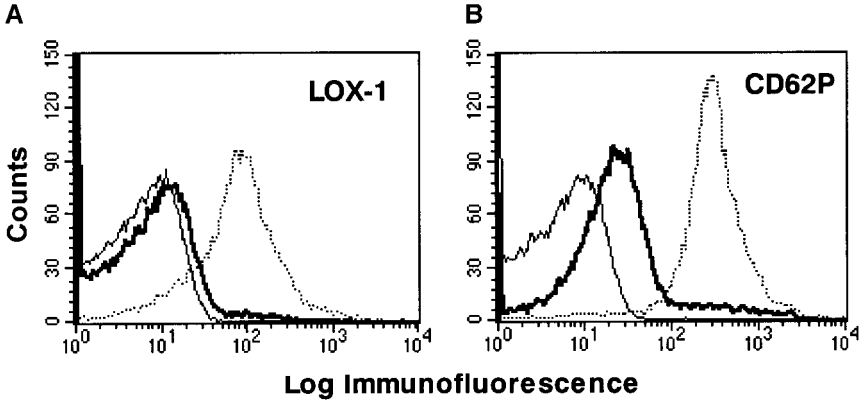

FIG. 3. Flow cytometry analysis showing the cell-surface expression of LOX-1 on human platelets. (A) Platelets were stained with anti-LOX-1 and isotype-matched control antibody as described under Materials and Methods, and analyzed by flow cytometry with the gating on the CD41 positive cells. (B) Staining with human CD62P as positive control. Representative immunofluorescence histograms are depicted (light lines indicate staining with control IgG; bold lines indicate staining with specific antibody for LOX-1 or CD62P in resting platelet, and dashed lines indicate thrombin activated platelets).

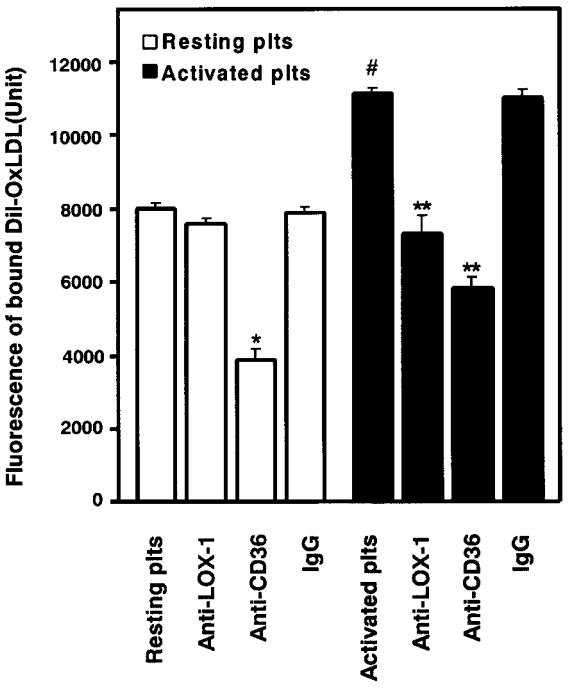

FIG. 4. Specific binding of DiI-OxLDL to human platelets: role of LOX-1. Freshly isolated resting and activated human platelets were incubated in suspension with 10 μg/mL of DiI-OxLDL for 3 h at room temperature. Preincubations with anti-CD36, anti-LOX-1 blocking antibodies, or isotype-matched control IgG were also performed. The binding of OxLDL to platelets was quantified by calculating the florescence of DiI in the labeled OxLDL. Nonspecific binding was defined as the binding that was not displaced by a 100-fold excess of unlabeled OxLDL, and was subtracted from each value. Values are the mean of three experiments in duplicate determinations ± SEM. Statistical analysis was performed with unpaired Student *t* test. $*P < 0.001$, compared with resting platelets; $\#P < 0.01$, compared with resting platelets; $**P < 0.001$ compared with activated platelets.

LOX-1 activity in platelets. Activity of some proteins in platelets is regulated by translocation from intracellular store granules to plasma membrane on the stimulation of platelets. Therefore, localization of LOX-1 before and after stimulation of platelets was analyzed. After stimulation by thrombin, platelets exposed the epitopes for anti-LOX-1 antibody on the plasma membrane, while unstimulated platelets showed minimal exposure (Fig. 3A). The parallel experiments detecting CD62P(P-selectin), which is well-known to translocate to plasma membrane on stimulation of platelets, showed similar results (Fig. 3B). This is confirming the fusion of store granules with plasma membrane actually occurred in the experiments, and suggesting the translocation of LOX-1 upon thrombin stimulation.

Then, we further analyzed the translocation of LOX-1 by evaluating the binding of OxLDL to platelets, in order to clarify the functional difference between CD36 and LOX-1. Figure 4 shows the specific binding of OxLDL to resting and activated platelets. The latter exhibited a moderate increase of the binding. In resting platelets, anti-CD36 antibody significantly attenuated the binding of OxLDL, whereas anti-LOX-1 antibody did not. In activated platelets, both anti-LOX-1 and anti-CD36 antibody significantly inhibited the binding. Importantly, anti-LOX-1 antibody inhibited most of the activation-dependent increase of the binding. These data revealed that CD36 is a major binding site for OxLDL in resting platelet, while LOX-1 appears to be critical in the activated platelets.

To verify the role of LOX-1 in platelets related to atherosclerosis, immunohistochemistry was performed to examine the expression of LOX-1 in an atheroma accompanying thrombosis (Fig. 5). Immunostaining of the lesion with anti-platelet glycoprotein IIb (CD41) antibody confirmed the presence of platelets. Immunostaining with antiLOX-1 antibody also showed diffuse distribution of LOX-1 positivity in the thrombus. Immunodouble-staining with both antibodies confirmed the co-localization of LOX-1 with platelets. These results indicated that LOX-1 is accumulated and exposed at thrombi, in which much activated platelets are aggregated.

DISCUSSION

Evidence is accumulating to support the importance of LOX-1 in the pathogenesis of atherosclerosis (21, 25). LOX-1 was initially identified as the major cell surface receptor for OxLDL in aortic endothelial cells (1). Shortly after, the expressions in macrophages and smooth muscle cells were reported (22, 26, 27). Immunohistochemical analyses revealed that LOX-1 was indeed accumulated in human and animal atherosclerotic lesions. Although the most prominent expression was seen in endothelial cell, moderate expressions in atheroma-derived macrophages and smooth muscle cells have also been observed (21, 25).

The expression of LOX-1 in circulating blood cells has not been reported. Many reports have suggested that OxLDL modulates platelet functions that are potentially implicated in the initiation and progression of atherosclerosis (7, 28, 29). When incubating with OxLDL, platelets are in a pre-activated state, which is more sensitive to further stimulation (11). OxLDL increases thrombin-induced platelet aggregation and serotonin release (30). We therefore, analyzed the expression of LOX-1 in platelets that might mediate the observed actions of OxLDL in platelets. The expression of LOX-1 in human platelets was identified as a major binding protein for OxLDL. The other part of the binding activity for OxLDL in platelets was ascribed to CD36, also called platelet glycoprotein IV (15–18). CD36 is a membrane glycoprotein expressed on monocytes, platelets, and certain microvascular endothelium serving as an receptor for thrombospondin, collagen, *Plasmodium falciparum*-infected erythrocytes, apoptotic cells and oxidized LDL (15, 31). However, no sequence homology between them was found (1). This

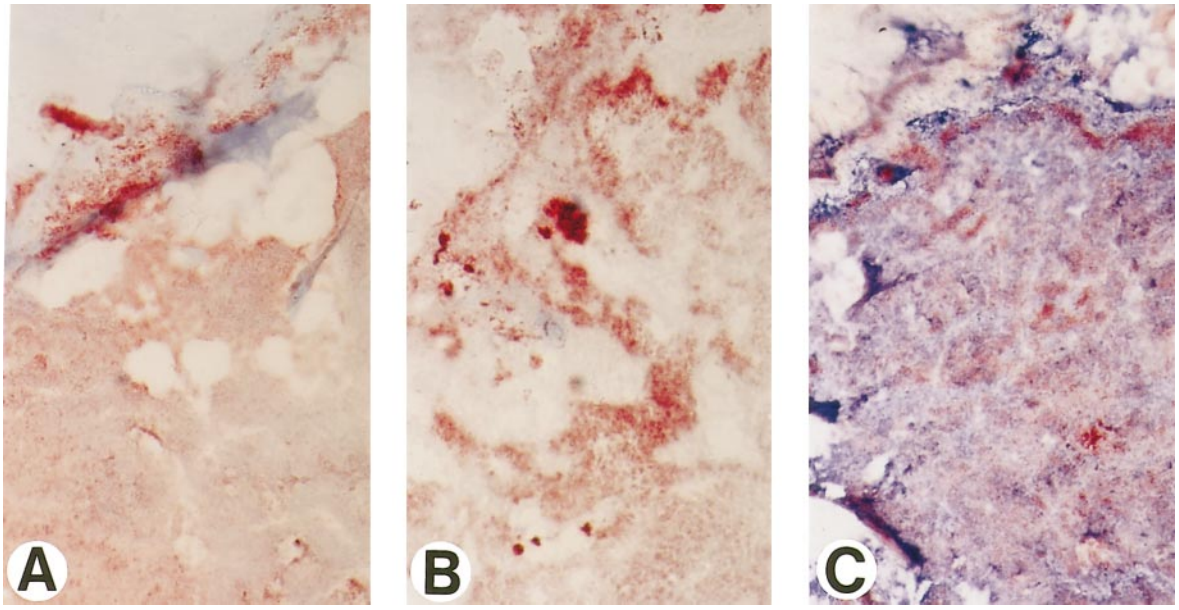

FIG. 5. Micrographs of a culprit lesion in a patient with unstable angina pectoris (UAP). Immunohistochemical staining of sections of advanced atherosclerotic lesion with thrombus formation. (A) Immunostaining for the platelet marker glycoprotein IIb reveals an atherosclerotic lesion containing an abundance of platelets. (B) Immunostaining for LOX-1 in a section adjacent to A. (C) In an additional adjacent section, immunodouble staining for platelet glycoprotein IIb (blue) and LOX-1 (red) reveals double staining (purple) of most of platelets, indicating LOX-1 positivity. Original magnification: ×533.

suggests that LOX-1 may function in some way distinct from CD36. In the present study, difference of the subcellular localization between these two receptors was observed. CD36 is present on plasma membrane even in resting platelets. LOX-1 appears on the surface of platelets on activation. This indicates that these two molecules work in different phase of platelets metabolism. Taking the activation-dependent exposure of LOX-1 protein into consideration, LOX-1 might work more in thrombi than in circulating resting platelets.

Once endothelium is injured, platelets are quickly activated and aggregated at the culprit site of blood vessel (7). This results in an accumulation of LOX-1 protein at the injured endothelium. The expression of LOX-1 in dysfunctioned endothelium has been postulated to initiate many vascular diseases (1). The presence of LOX-1 in platelets and rapid delivery to the lesions may further accelerate the pathological changes in the blood vessel involved.

It should be noted that LOX-1 can recognize and bind activated platelets (5, 6). Cross-linking of activated platelets by LOX-1 exposed on the activated platelets surface may stabilize thrombi, leading to the formation of larger thrombi or promoting hemostasis in arteries. This possibility is a critical issue needs to be investigated. Our preliminary results do support the importance of LOX-1 in thrombotic process.

In summary, we demonstrate that LOX-1 is expressed in human platelets and megakaryocytic cell lines. Further characterization of the functions of LOX-1 in platelets would provide novel insights into the pathogenesis of atherosclerosis and thrombosis.

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