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Lectin-like oxidized low-density lipoprotein receptor (LOX-1) in sickle cell disease vasculopathy

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ABSTRACT

Lectin-like oxidized low-density lipoprotein (LDL) receptor-1 (LOX-1) is an endothelial receptor for oxidized LDL. Increased expression of LOX-1 has been demonstrated in atherosclerotic lesions and diabetic vasculopathy. In this study, we investigate the expression of LOX-1 receptor in sickle cell disease (SCD) vasculopathy. Expression of LOX-1 in brain vascular endothelium is markedly increased and LOX-1 gene expression is upregulated in cultured human brain microvascular endothelial cells by incubation with SCD erythrocytes. Also, the level of circulating soluble LOX-1 concentration is elevated in the plasma of SCD patients. Increased LOX-1 expression in endothelial cells is potentially involved in the pathogenesis of SCD vasculopathy. Soluble LOX-1 concentration in SCD may provide a novel biomarker for risk stratification of sickle cell vascular complications.

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1. Introduction

Sickle cell disease (SCD) is associated with significant morbidity and mortality due, in large measure, to vascular injury and occlusion [1]. Apart from extensive hemolysis, SCD is characterized by recurrent vaso-occlusive episodes or "crises" manifested by severe bone pain, acute chest syndrome, priapism, and stroke, as well as chronic irreversible damage to the heart, brain, lungs, skin, kidneys, spleen and femoral heads [2]. Cumulative acute on chronic tissue injury results in significant morbidity and early mortality.

Inflammation and abnormal adhesion of sickle red blood cells (RBCs), leukocytes and platelets to the vascular endothelium are postulated to play a central role in the pathogenesis of vasculopathy associated with sickle cell disease (SCD) [3,4]. Endothelial cells in SCD display vasoconstrictive, proinflammatory and prothrombotic changes [1,5,6]. Sickle RBCs may initiate damage through activation of the endothelium mediated by cell surface adhesion molecules such as vascular cell adhesion molecule (VCAM), intercellular adhesion molecule (ICAM), platelet endothelial cellular adhesion molecule (PECAM-1; CD31), E-selectin, and P-selectin [7,8]. Levels of the soluble forms of these adhesion molecules are elevated in patients with SCD, especially during crisis [9], which may lead to cellular aggregates in the postcapillary venule and vaso-occlusive painful crisis [10].

Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) has been identified as a major receptor on endothelial cells for oxidized low-density lipoprotein (ox-LDL) as well as for the surface phosphatidylserine of senescent RBCs [11,12]. LOX-1 can bind and internalize ox-LDL [11], senescent RBCs [13], apoptotic cells [13], activated platelets [14] and neutrophils [15]. This, in turn, induces endothelial damage and promotes the atherogenic process [16,17]. Enhanced expression of LOX-1 is associated with and has been implicated in atherosclerosis and diabetic vascular disease [16,18–20]. High levels of soluble LOX-1 were observed in patients with acute stroke [21]. The pathogenesis of sickle cell vasculopathy shares some of the pathological features seen during the initiation of atherosclerosis. Based on our previous observations concerning endothelial dysfunction initiated through the LOX-1 receptor [16], we hypothesize that LOX-1 may be an important mediator in the pathophysiology of sickle cell vaso-occlusive events.

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2. Material and methods

2.1. Patient recruitment and study design

The University of California Davis Institutional Review Board approved the study and a written informed consent was obtained from all patients or from their parents or guardians. Sickle cell anemia (HbSS) was confirmed by hemoglobin electrophoresis and high performance liquid chromatography (HPLC). The comparator group (HbAA) included healthy individuals with normal hemoglobin electrophoresis recruited at the same institution, matched for age, sex, and ethnicity. Pediatric (ages 6–18 y) and adult (ages 27–58 y) patients were recruited from the Sickle Cell Clinic at the University of California Davis Medical Center (UCDMC) [22]. SCD patients on chronic transfusion, those treated with hydroxyurea in the past two weeks, with a history of malignancy, diabetes mellitus, systemic arterial hypertension, other vascular or connective tissue disorders, and pregnancy were excluded from the study. Venous blood samples (10 ml) were collected in sodium citrate, and platelet-free plasma was immediately separated and stored frozen at −20 °C.

2.2. RBC binding assay

Human coronary endothelial cells (HCECs) were purchased from Clonetics. Human brain microvascular endothelial cells (HBMVECs — ACBRI 376) and culture media were purchased from Cell Systems. Cells were cryopreserved at second passage and cultured at <5 passages for all studies. Chinese hamster ovary (CHO) cells stably expressing human LOX-1 (hLOX-1-CHO) and wild-type CHO-K1 cells were maintained as described previously [13]. Sickle red blood cells (RBCs) from SCD patients and normal controls were collected, washed three times with PBS and re-suspended at 20% hematocrit in PBS containing 0.1% glucose. Some normal RBCs were heat-damaged by incubating them at 49.5 °C for 20 min. RBCs (adjusted to a hematocrit of 1%) were incubated with HCEC, CHO-K1 cells, or hLOX-1-CHO cells in culture media at 37 °C for 3 h. Unbound RBCs were removed by extensive gentle washing with media, and the cells were fixed with PBS containing 4% formaldehyde and stained with Giemsa [13].

2.3. Real-time quantitative RT-PCR assay for LOX-1 mRNA

Human brain microvascular endothelial cells (HBMVECs) were cultured with Complete Classic Medium Kit with Serum and Culture Boost (Cell System) to confluency. Cells were incubated with RBCs (isolated from SCD patients or normal controls) for 15 min to 18 h and washed 3 times with medium. RNA was isolated using the RNeasy kit (QIAGEN) according to the manufacturer's instruction. cDNAs were prepared using QuantiTech Reverse Transcription Kit (QIAGEN). Quantitative real-time PCR to detect LOX-1 gene expression was performed using SYBR Green incorporation on Stratagene Mx3005 system using the following human LOX-1 primer pair: forward primer 5′-*GCGACTCTAGGGGTCCTTTG*-3′, reverse primer 5′-*GTGAGTTAGGTTTGCTTGCTCT*-3′; Quantitation of mRNA expression was performed by the comparative Ct method, and normalized to the human Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) gene using the following primer pair: forward primer 5′-CTGGGCTACACTGAGCACC-3′, and reverse primer 5′-AAGTGGTCGTTGAGGGCAATG-3′.

2.4. Immunofluorescent and immunohistochemistry staining

For immunofluorescent staining: cultured HBMVECs were incubated with RBCs (isolated from SCD patients or normal controls) for 6 h, washed 3 times with medium, fixed with 4% paraformaldehyde for 20 min at room temperature, blocked with 20% donkey serum for 30 min at RT, then incubated with primary antibodies mouse anti-human CD31(pre-diluted, Dako) and chicken anti-human LOX-1(10 μg/ml, HUC52) overnight at 4 °C, followed by incubation with secondary antibodies Cy3-donkey anti-mouse and FITC-donkey anti-chicken (1:500, Jackson ImmunoResearch Lab) for 1 h at RT. Nuclei were stained with DAPI and images were acquired using a Keyence microscope. Immunohistochemistry staining of LOX-1 was performed on formalin-fixed paraffin-embedded human tissues collected from an autopsy of a sickle cell disease patient who died in sickle cell crisis and a non-sickle cell disease control autopsy. The slides were immunostained after heat-induced antigen retrieval using rabbit polyclonal antibody to human LOX-1 (5 μg/ml, Novus Biologicals CO, USA) as previous described [18].

2.5. Soluble LOX-1 measurement

Plasma circulating soluble LOX-1 (sLOX-1) levels were measured using a commercially available enzyme-linked immunosorbent assay kit (Adipo Bioscience; Santa Clara, California) as described previously [12,23].

3. Results and discussion

3.1. Evidence of red blood cell binding to LOX-1 on endothelial cells in vitro

Adhesion and phagocytosis of sickle RBCs was noted in cultured human coronary endothelial cells (HCECs; Fig. 1A). Also, overexpression of LOX-1 in CHO cells resulted in binding SCD RBCs, which was blocked by LOX-1 antibody (Fig. 1B). Our findings indicate that sickle RBCs adhere to endothelial cells as well as to CHO cells engineered to express human LOX-1. Under *in vivo* conditions during sickle cell crisis, hypoxia results in ATP depletion and hemoglobin S polymerization, leading to red blood cell sickling and exposure of phosphatidylserine (PS) on the outer surface of the plasma membrane [10].

3.2. Evidence of LOX-1 gene expression in endothelial cells induced by sickle cells in vitro

LOX-1 mRNA expression in human endothelial cells was significantly increased following incubation with sickle RBC compared with normal RBC (Fig. 2A). Expression of LOX-1 protein was also prominently up-regulated in endothelial cells incubated with sickle RBC (Fig. 2B). Expression of upregulated LOX-1 could be a marker for endothelial dysfunction.

3.3. Detection of soluble LOX-1 (sLOX-1) in SCD patient plasma

Plasma samples from 22 SCD patients and 9 healthy control subjects were assayed for sLOX-1. The concentration of circulating sLOX-1 protein in plasma of SCD patients (3.92 ± 2.35 ng/ml) was significantly higher ($p = 0.0039$) than in control healthy subjects (1.40 ± 0.60 ng/ml) (Fig. 3A). It is possible that the plasma concentration of sLOX-1 correlates with clinical status such as impending sickle crisis. Investigation of this question lay beyond the scope of the present study but will be explored in future studies, since it is possible that changes in circulating sLOX-1 levels may serve as a harbinger of impending sickle cell crisis.

3.4. Evidence of LOX-1 protein expression in endothelial cells in vivo

Autopsy findings of a SCD patient (40 year-old female) who died in painful vaso-occlusive crisis (VOC) revealed histological features of vaso-occlusive microthrombi formation. H&E stain of midbrain tissue showed thrombus formation and occlusion of vessels. Expression of LOX-1 in brain vascular endothelial cells was confirmed by immunohistochemical staining, whereas the expression of LOX-1 was not detected in normal control brain tissue (Fig. 3B). Similar results were obtained in SCD heart tissue (data not shown). Vaso-occlusive damage caused by

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