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Lactate dehydrogenase-A is indispensable for vascular smooth muscle cell proliferation and migration

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ABSTRACT

The proliferation and migration of vascular smooth muscle cells (VSMCs) have been implicated in the pathogenesis of atherosclerosis. Increased aerobic glycolysis is a key feature of cellular phenotypes including cancer and immune cells. However, the role of aerobic glycolysis in the atherogenic phenotype of VSMCs remains largely unknown. Here, we investigated the role of lactate dehydrogenase-A (LDHA), which is a key enzyme for glycolysis, in the proliferation and migration of VSMCs. Activation of primary rat VSMCs with fetal bovine serum (FBS) or platelet-derived growth factor (PDGF) increased their proliferation and migration, glycolytic activity, and expression of LDHA. Wound healing and transwell migration assays demonstrated that small interfering RNA-mediated knockdown of LDHA and pharmacological inhibition of LDHA by oxamate both effectively inhibited VSMC proliferation and migration. Inhibition of LDHA activity by oxamate reduced PDGF-stimulated glucose uptake, lactate production, and ATP production. Taken together, this study shows that enhanced glycolysis in PDGF- or FBS-stimulated VSMCs plays an important role in their proliferation and migration and suggests that LDHA is a potential therapeutic target to prevent vessel lumen constriction during the course of atherosclerosis and restenosis.

1. Introduction

Characteristic metabolic reprogramming in proliferative cells has emerged as a crucial player in determination of their phenotypes and signal transduction processes [1]. Although mainly studied in cancer models, growth factors influence cell proliferation by altering intracellular metabolism; therefore, several key enzymes that regulate glycolysis or mitochondrial respiration have been proposed as targets for the treatment of cancer [2,3]. A Warburg-like increase in the glycolytic rate also appears to be inherent to non-malignant cells such as immune and skeletal muscle cells [4–6]. Several recent studies suggested that metabolic reprograming in vascular smooth muscle cells (VSMCs) participates in the complex regulation of their proliferation and migration [7–9].

VSMC proliferation and migration are involved in physiological processes such as development, wound healing, and angiogenesis [10]. However, once tissue repair is complete, the continuation of VSMC proliferation and migration due to excessive mitogens such as growth factors in the cellular microenvironment induces pathogenic vessel lumen constriction during the course of atherosclerosis and restenosis [11,12]. For this reason, extensive research has focused on elucidating the intracellular mechanisms involved in the regulation of VSMC proliferation and migration [13–15]. Recent studies showed that enhanced glycolysis may be required for

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platelet-derived growth factor (PDGF)-induced VSMC proliferation, supported by increased glycolytic enzyme expression, lactate production, and glucose utilization [7,8].

Modulating VSMC metabolism via anti-glycolytic therapies may be a means to treat atherosclerosis and restenosis because increased glycolysis is associated with the proliferation of VSMCs and cancer cells [8]. The enzyme lactate dehydrogenase-A (LDHA) is of particular interest because it catalyzes the nicotinamide adenine dinucleotide-dependent reduction of pyruvate to lactate, an essential step for regenerating nicotinamide adenine dinucleotide, which is needed to maintain glycolysis and other metabolic activities [16–18]. However, the role of LDHA in VSMC proliferation and migration has not been investigated. Therefore, we aimed to examine the role of LDHA in the proliferation and migration of VSMCs.

2. Material and methods

2.1. Immunohistochemical staining of balloon-injured rat carotid arteries

The rat carotid artery balloon-injury method was described previously [19]. Rat arterial tissue was fixed in 4% paraformaldehyde and embedded in paraffin. For immunohistochemical staining, paraffinized aorta sections were deparaffinized with xylene and ethanol. Endogenous peroxidase was blocked by treatment with 3% H_2O_2 for 15 min and then samples were incubated with an anti-LDHA antibody (Cell Signaling Technology, 1:100). Blocking of endogenous peroxidase and protein detection were performed using an Ultravision LP Detection System HRP Polymer kit (Lab Vision, Fremont, CA, USA) according to the manufacturer's protocol.

2.2. Cell culture

Rat aortic smooth muscle cells were cultured using a transplant method as described previously [20]. Cells were isolated from a 4-week-old Sprague-Dawley male rat (Hyochang Science, Daegu, Korea). The trimmed aorta was washed with sterilized cold phosphate-buffered saline (PBS) and sliced into pieces measuring 1–3 mm^2. The pieces of aorta were attached to dishes and cultured in low-glucose Dulbecco's Modified Eagle Medium (Gibco, Grand Island, NY, USA) supplemented with 20% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) for 2 weeks at 37 °C in 5% CO_2. The medium was changed every day. Cells were maintained in Dulbecco's Modified Eagle Medium containing 5.5 mM glucose and supplemented with 10% FBS. Cells at passage 4–6 were used in all experiments.

2.3. Western blot analysis

Cells were lysed in lysis buffer containing 20 mM Tris-HCl (pH 7.4), 5 mM EDTA (pH 8.0), 10 mM $Na_4P_2O_7$, 100 mM NaF, 2 mM Na_3VO_4, 1% NP-40, and protease and phosphatase inhibitors. Protein samples were separated on a 10% SDS-PAGE gel and transferred to a PVDF membrane. Membranes were blocked with 5% skimmed milk prepared in Tris-buffered saline containing 0.1% Tween 20 (TBST) and incubated with each primary antibody overnight at 4 °C. The primary antibody against LDHA (1:1000) was purchased from Cell Signaling Technology (Beverly, MA, USA), and that against β-actin (1:5000) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Membranes were thrice washed with TBST and incubated with a HRP-conjugated mouse (Santa Cruz) or rabbit (Cell Signaling Technology) secondary antibody. HRP was detected using ECL reagent (Bionote, Suwon, Gyeonggido, Korea).

2.4. LDHA activity

LDHA activity was determined by measuring the consumption of NADH in 20 mM HEPES-K^+ (pH 7.2), 0.05% bovine serum albumin, 20 μm NADH, and 2 mM pyruvate using a microplate reader (excitation, 340 nm; emission, 460 nm) as previous described [21,22].

2.5. siRNA transfection

For gene silencing, cells were transfected with 50 nM scrambled siRNA or siLDHA (Bioneer, Daejeon, Korea) using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

2.6. Cell counting

Non-transfected VSMCs and those transfected with scrambled small interfering RNA (siRNA) or siRNA targeting LDHA (siLDHA) for 24 h were serum-starved for 18 h and incubated with 10% FBS or 20 ng/ml rat recombinant PDGF (R&D Systems, Minneapolis, MN, USA) with or without 60 mM sodium oxamate (Sigma, St. Louis, MO, USA). Cells were trypsinized, stained with trypan blue solution, and counted with a hemocytometer.

2.7. Migration assay

For the wound healing assay, a wound was generated by scratching VSMCs with a 200 μl pipette tip. When the wound had closed, cells were washed thrice with PBS, fixed in 4% paraformaldehyde, washed thrice with PBS, and stained with 0.05% crystal violet. For the transwell migration assay, cells were seeded in a membrane transwell with a pore size of 8 μm (Corning Incorporated, New York, NY, USA). After being washed thrice with PBS, cells were fixed in methanol. Cells inside the transwell were removed with a swab. Migrated cells were washed thrice with PBS, stained with Mayer's hematoxylin (Sigma), washed with PBS, and dried completely.

2.8. Glucose uptake, lactate, and ATP assays

Glucose uptake was measured using a Glucose Uptake Colorimetric Assay Kit (Biovision, Milpitas, CA, USA). Lactate and ATP were measured using assay kits (Bioassay Systems, Hayward, CA, USA) according to the manufacturer's protocol.

Determination of the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR).

Primary VSMCs were seeded in a Seahorse XF24 plate, serum-starved for 18 h, and incubated with or without PDGF or sodium oxamate for 24 h. Then, the basal extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were measured and automatically calculated using a Seahorse XF24 analyzer (Seahorse Bioscience, North Billerica, MA, USA).

2.9. Statistical analysis

All values are presented as the mean ± SEM. Statistical analysis was performed using the two-tailed Student's t-test. $p < 0.05$ was considered statistically significant.

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