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Hypoxia triggers angiogenesis by increasing expression of LOX genes in 3-D culture of ASCs and ECs

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ABSTRACT

Objectives: This study aimed to investigate the expression changes of LOX (lysyl oxidase) family genes, *VEGFA*, and *VEGFB* under hypoxic conditions in a co-culture system of ASCs (adipose-derived stromal cells) and ECs (endothelial cells).

Materials and methods: ASCs and ECs were co-cultured under hypoxic and normal oxygen conditions in a 1:1 ratio, and the formation of vessel-like was detected at 7 days. The transwell co-culture system was used and cell lysates were collected at 7 days after co-culture in hypoxic and normal oxygen condition. Semi-quantitative PCR was performed to quantify the mRNA expression of *VEGFA*, *VEGFB*, and the LOX genes (*LOX*, *LOXL-1*, *LOXL-2*, *LOXL-3*, and *LOXL-4*). Expression changes were determined by densitomery.

Results: Enhanced angiogenesis was detected in the co-culture of ASCs and ECs under hypoxic conditions. Among the genes screened, *VEGFA*, *VEGFB*, *LOXL-1*, and *LOXL-3* in ECs, both mono-cultured and co-cultured, were significantly enhanced after culturing under hypoxic conditions. In ASCs, *VEGFB*, *LOXL-1*, and *LOXL-3* were upregulated.

Conclusions: Contact co-culture between ASCs and ECs promotes angiogenesis under hypoxia. *LOXL-1* and *LOXL-3* expressions were increased in both ASCs and ECs and might play important roles in the enhanced angiogenesis promoted by hypoxia.

1. Introduction

So far, the significance of lysyl oxidase (LOX) proteins expressed in head and neck squamous cell carcinoma (HNSCC) is still controversial. Therefore, many studies were conducted to explore the relationship between the *LOX* genes and tumors, cancers, and carcinomas whose expansion and growth are related to angiogenesis. The LOX family consists of five members: *LOX, LOXL-1* (lysyl oxidase-like 1), *LOXL-2* (lysyl oxidase-like 2), *LOXL-3* (lysyl oxidase-like 3), and *LOXL-4* (lysyl oxidase-like 4). Moreover, the LOX family proteins are amine oxidases and their secretion is copper-dependent [1]. LOX family members are known to be upregulated by a variety of external factors including *TGF-1*, hypoxia, and mechanical stress [2]. In oral squamous cell carcinoma, some studies suggest that *LOX* is upregulated [3] and is essential for hypoxia-induced metastasis in HNSCC [4], whereas other reports show that *LOX* and *LOXL-2* are downregulated in head and neck cancer [5].

The gene expression of LOX family members in adipose-derived stromal cells (ASCs) and endothelial cells (ECs) under hypoxic conditions is still unknown. The LOX family is considered a significant player in tumor metastasis [6]. There is a complex connection between metastasis and angiogenesis [7,8]. Moreover, increasing studies are reporting that the LOX family promotes angiogenesis [2]. Since enhanced angiogenesis is observed under hypoxic condition, this study explores the relationship between hypoxia and the LOX family.

Conventional cell-culture is carried out at atmospheric condition with about 20% oxygen. However, in their natural microenvironment,

Abbreviations: ASCs, adipose-derived stromal cells; CLSM, confocal laser scanning microscopy; DAPI, 49,6-diamidino-2-phenylindole; ECM, extracellular matrix; ECs, endothelial cells; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; IRB, Institutional Review Board; LOX, lysyl oxidase; LOXL1, lysyl oxidase-like 1; LOXL3, lysyloxidase-like 3; LOXL4, lysyloxidase-like 4; PBS, phosphate-buffered saline; TGF-1, transforming growth factor-1; VEGFA, vascular endothelial growth factor A; VEGFB, vascular endothelial growth factor B

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cells are maintained at low oxygen tensions; for instance, the oxygen concentration of arterial blood is about 12% whereas that in tissues is about 3% and varies at different locations [9]. As mentioned above, it is therefore necessary to explore the cellular properties under hypoxic conditions.

Mounting evidence demonstrates that hypoxia plays a positive role in tumor angiogenesis [10]. Many studies use hypoxia to study the mechanism of angiogenesis for preventing tumor growth [11]. Meanwhile, it is well known that angiogenesis is essential for creating functional tissues and organs in tissue engineering. Therefore, development of vascularization as a supplement of nutrition is becoming a key challenge in tissue engineering.

In a previous study [12], we found that hypoxia promotes formation of vascular structures in ECs and ASCs cultured in 3-D gels as observed by confocal laser scanning microscopy. However, the mechanism underlying this result is still unknown. Among numerous reports about tumor therapy, *LOX* is reported to regulate tumor metastasis [13,14]. Furthermore, it is involved in the modification of extracellular matrix (ECM) [1]. Since tumors are full of blood vessels and show the ability of metastasis, the role of *LOX*, which is relevant to hypoxia and angiogenesis, should also be explored.

2. Methods and materials

2.1. Cell culture

The animal materials used in this study were obtained in accordance with ethical principles. The protocol was examined and approved by the Institutional Review Board (IRB). The methods used to obtain the ASCs and ECs have been described in our previous study [12]. The methods used to generate green fluorescent protein (GFP)-positive ASCs and DsRed-Express-positive ECs are also described in our previous study [12].

2.2. Mono-culture and co-culture at normal oxygen and hypoxia

The angiogenesis assay was carried out in a 3-D gel model. The GFP-ASCs and RFP-EC were mixed in a 1:1 ratio and then divided into two groups, one was placed under normal oxygen conditions (the oxygen concentration was set as 20%), and the other was placed under hypoxic condition (the oxygen concentrations was set as 2%). These two groups of cells were transferred into the 3-D gel to proliferate and were cultured for 1 week.

To determine the gene profile of the LOX family in ASCs and ECs both under normal and hypoxic conditions, the transwell co-culture system was also used in this study (Fig. 1). This system is also described in our previous study [12]. Finally, the cell lysates (1000 μl) of ECs and ASCs were collected at 1, 3, 5, and 7 days after both mono-culture and co-culture under normal and hypoxic conditions.

2.3. Immunofluorescence staining

After 2 days of co-culture, the transwell insert was removed. The ASCs or ECs seeded in six-well plates were washed thrice with PBS, fixed with 4% paraformaldehyde for 15 min, rinsed thrice with PBS, and then treated with 0.5% Triton X-100 in PBS for 25 min. The cells were blocked with 5% bovine serum albumin for 60 min before incubation with the primary antibodies targeting the LOX family proteins at proper dilutions as suggested by the manufacturer. After 90 min of incubation, the cells were rinsed thrice with PBS and incubated with counter secondary antibody to target LOXs family. After 15 min of incubation, the cells were rinsed thrice with PBS, and then stained with 4,6-diamidino-2-phenylindole (DAPI) for 10 min. The images were captured using confocal laser scanning microscopy (CLSM) (Olympus, Tokyo, Japan).

2.4. Western blot analysis

ASCs and ECs from the four groups were washed thrice with ice-cold PBS and the cells were harvested and lysed using lysis buffer containing protease inhibitors. The collected lysates were centrifuged at 10,000×*g* at 4 °C for 5 min. The supernatant was then collected and the bicinchoninic acid assay was used to measure protein concentrations. The protein samples were then solubilized and boiled in SDS sample buffer for 5 min and then separated using 10% SDS-PAGE at 100 V for 90 min. The separated proteins were transferred to a polyvinylidene fluoride membrane. The membranes were incubated in blocking solution containing 5% BSA in TBST for 1 h at room temperature (about 25 ℃) and then incubated overnight at 4 °C with the primary antibodies against GAPDH, LOX, LOXL-1, LOXL-2, LOXL-3, LOXL-4, VEGFB, and vinculin. The membrane was then washed slightly and probed with the respective secondary antibodies for 60 min at room temperature. After washing thrice with TBST, the chemiluminescence detection system was used to visualize and record the binding. The expression levels of the targeted proteins were compared to those of the control based on relative band intensities.

2.5. Semi-quantitative polymerase chain reaction (PCR)

The RNA samples of ASCs and ECs from the four groups were obtained using the RNeasy Plus Mini Kit (Qiagen, CA, USA) with a genomic DNA eliminator. This method has also been described in our previous study [12]. The samples were used to determine the expression change of the LOX family genes along with *VEGFA* and *VEGFB* (Table 1).

2.6. Statistical analysis

All experiments were conducted at least three times independently,

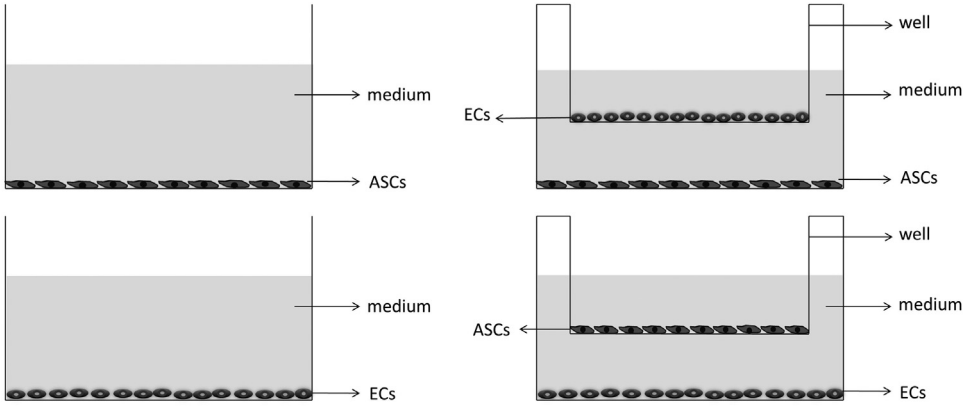

Fig. 1. The mono-culture and co-culture model system.