2023 45th Annual International Conference of the IEEE Engineering in Medicine & Biology Society (EMBC) | 979-8-3503-2447-1/23/$31.00 ©2023 IEEE | DOI: 10.1109/EMBC40787.2023.10340043

Evaluation of the effects of glucose and oxygen on the vascular endothelial cell migration

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Abstract— **Glucose is essential as the main energy source for living organisms. However, excessive elevation of blood sugar levels can lead to diabetes and serious complications such as arteriosclerosis. Even though blood sugar levels as well as hypoxia associated with hyperglycemia are known to be closely related to diabetes complications, the responses of vascular endothelial cells to glucose and oxygen have not been fully investigated. In this study, using a microfluidic device that can control the oxygen concentration, we observed the behavior of vascular endothelial cell monolayers while simultaneously controlling glucose and oxygen levels. Results showed that the cell migration speed was increased by high-glucose exposure in an oxygen-rich environment, but was decreased in a hypoxic environment regardless of glucose condition. The expression of vascular endothelial-cadherin at the cell periphery, which plays a role in cell-cell adhesion, was increased by hypoxic exposure, but was largely independent of glucose condition. This suggested that cell-cell adhesion is less involved in the increase in migration caused by high glucose. Furthermore, stabilization and nuclear translocation of hypoxia-inducible factor-1α, which is involved in cellular hypoxia sensing, increased 5 h after exposure to high glucose, but decreased 3 days after the exposure. This indicated that intracellular hypoxia was generated by increased oxygen consumption in mitochondria just after the high-glucose exposure, but it was moderated within 3 days.**

I. Introduction

Most of the carbohydrates obtained through the diet are digested and eventually become glucose, which is used as an energy source. In healthy individuals, the blood sugar level is 4 mM in a fasting state and 7 mM in a postprandial state [1]. Chronic hyperglycemia can lead to diabetes due to the insufficient action of insulin, resulting in serious complications. The effects of oxidative stress on vascular endothelial cells with elevated blood sugar levels can cause damage to the vascular endothelium, thereby allowing cholesterol and monocytes to enter the vascular wall, and their debris to form plaques, leading to arteriosclerosis [2]. When arteriosclerosis blocks blood vessels, oxygen and nutrients are not distributed to tissues *in vivo*, leading to stroke and ischemic heart disease. Hence, glucose and oxygen conditions in blood are related to diabetes complications [3]. However, few studies have considered the combined effect of glucose and oxygen on vascular endothelial cells. Elucidation of their impact on the behavior of vascular endothelial cells could provide insights to prevent and treat diabetes-related diseases.

We have developed a microfluidic device capable of controlling the oxygen concentration in the microenvironment, and studied the hypoxic response of vascular endothelial cells [4,5,6]. The device mainly consists of polydimethylsiloxane (PDMS), a polymer compound with high transparency and gas-permeability. A monolayer of vascular endothelial cells was formed in the device, and the oxygen concentration around the cells was controlled by gas exchange through supplying gas mixtures at a pre-adjusted oxygen concentration to the two gas channels in the device [4,5]. The study results showed that the collective migration of vascular endothelial cells was oxygen-dependent. The migration speed peaked under a hypoxic condition of about 1.7% O_2, and decreased under extremely low oxygen concentrations [5]. Simultaneously, it was observed that the nuclear translocation of hypoxia-inducible factor (HIF)-1α significantly increased at below 1.7% O_2. However, in the previous study, the glucose concentration in the cell culture medium was set at a constant value (5.5 mM) normally used for cell culture. Therefore, variation in oxygen-dependent cell migration according to glucose concentration was unknown.

The purpose of this study was to elucidate changes in the cell dynamics of vascular endothelial cells in response to alterations in glucose and oxygen concentrations. Using the oxygen concentration-controllable microfluidic device and adjusting the glucose concentration in the cell culture medium, we observed the dynamics of vascular endothelial monolayers formed in the media channels inside the device. Time-lapse observation of the vascular endothelial cells was performed at different oxygen and glucose concentrations. By analyzing the sequential phase-contrast microscopy images of the vascular endothelial monolayer, the speed of collective cell migration was measured. Further, 5 h after the start of experiments, the cells were fixed, and the relative area of VE-cadherin to the total cell area and the localization of HIF-1α were evaluated by immunofluorescence staining.

II. Materials and Methods

A. Microfluidic Device

Microfluidic devices of a square shape (each side is 30 mm, height is 3 mm) were used (Fig. 1) [4]. The device was fabricated using PDMS (Sylgard 184 Silicone Elastomer Kit, Dow Corning, USA), polycarbonate (PC) film, and cover glass. In the device, two gas channels (2 mm wide) were arranged at a height of 500 μm from the bottom to be perpendicular to three parallel media channels (2 mm wide). The oxygen concentration in the device was controlled by supplying gas mixtures at predefined oxygen concentrations to the two gas

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channels. In addition, to prevent the infusion of oxygen from the atmosphere surrounding the device, a 500-μm-thick PC film was embedded at a position of 1 mm in height in the device.

The device was made by the following process. PDMS was poured on each silicon wafer with the patterns of the media and gas channels, which were laminated by photolithography, to a thickness of 0.5 mm and cured in an oven at 60°C for >4 h. Then, additional PDMS was poured on top of the cured PDMS layer of the gas channel to a thickness of 2.5 mm, the PC film with holes at the positions of the inlets and outlets for all channels was submerged, and the PDMS was cured in the oven overnight. The cured PDMS layers of the media and gas channels were peeled off from the silicon wafer, and cut into squares (each side, 30 mm). The PDMS layers were sterilized, plasma-treated, and bonded together to form the gas channels. Furthermore, the PDMS layer and the glass cover slip were plasma-treated and bonded together to form the media channels. Immediately after the bonding, poly-D-lysine (P7886, Sigma-Aldrich, USA) solution was injected into the media channels, and the device was placed in an incubator (5% CO_2, 37°C) overnight. The media channels were then washed twice with sterilized water. Before cell seeding, the surface of the media channels was coated with 50 μg/ml fibronectin (FC010, Millipore, USA) to further enhance the cell adhesiveness.

B. Cell Culture

Human umbilical vein endothelial cells (HUVECs, C2519A, Lonza, Switzerland) were used for cell experiments. HUVECs were cultured with cell culture medium (EGM-2 BulletKit, CC-3162, Lonza) on a dish in an incubator (5% CO_2, 37°C). Cell suspensions of 2.5×10^6 cells/ml were prepared by harvesting HUVECs from the dish before reaching confluence, and 25 μl of each cell suspension was injected into the media channels of the device. After a 30-min incubation, the cell culture medium was replaced. Then, by culturing for 3 days while changing the cell culture medium every day, a monolayer of HUVECs covering the media channel was formed.

In this study, to investigate the effects of glucose conditions on HUVECs, the cells were exposed to a high-glucose environment by using a cell culture medium supplemented with glucose. EGM-2 containing 5.5 mM glucose was used as the control medium, and EGM-2 supplemented with 30 mM D-glucose (G0048, Tokyo Chemical Industry, Japan) was used for high-glucose experiments. In addition, in order to simulate both acute and chronic hyperglycemia, experiments were conducted by exchanging the control medium with the high-glucose medium at two different time points: just before or 3 days before (30 min after cell seeding in the device) the start of the experiment.

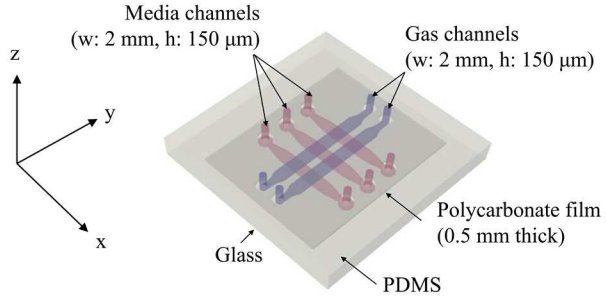

Figure 1. Schematic of the microfluidic device [4].

C. Measurement of Collective Cell Migration

The device with the HUVEC monolayer was placed in a stage incubator (INUBSF-ZILCS, Tokai Hit, Japan) (5% CO_2, 37°C) mounted on an inverted microscope (EVOS FL, Cell Imaging System, Life Technologies, USA). During the experiments, a steady uniform oxygen state was generated in the device by supplying a gas mixture to the two gas channels. A gas blender (3MFC GAS MIXER, KOFLOC, Japan) was used to adjust the oxygen concentration in the gas mixtures, maintaining 5% CO_2 and balancing with nitrogen. First, a gas mixture of 21% O_2 was supplied to the gas channel for 1 h for equilibrium. Next, the gas mixture of 21% O_2 was continued to be supplied for the normoxic condition N, or switched to 1% O_2 for the hypoxic condition H1. Here, the oxygen concentrations generated in the media channel by the conditions N and H1 were 21% and 1.3% O_2, respectively [3]. Phase-contrast microscopy images were then acquired every 10 min for 5 h. Using the Particle Image Velocimetry (PIV) software (JPIV) [7] and microscopy images taken every 20 min, migration velocities of vascular endothelial cells were analyzed. A region of interest (ROI) of 1,280×512 pixels (1,100×440 μm) was set near the center of the channel away from the sidewall of the media channel. The migration speed was quantified based on the displacement of subdivided ROIs of 32×32 pixels (27.5×27.5 μm). Experiments were performed using four devices for each condition.

D. Immunofluorescence Staining

After time-lapse observation, the cells were fixed using 4% paraformaldehyde/phosphate buffer (163-20145, Wako Pure Chemical Industries, Japan) to observe vascular endothelial (VE)-cadherin and HIF-1α by immunofluorescence staining. Anti-VE-cadherin (sc-9989, Santa Cruz Biotechnology, USA) and anti-HIF-1α (ab51608, Abcam plc, UK) were used at a dilution of 1:100 in PBS as primary antibodies, and Alexa Fluor 594 goat anti-mouse antibody (A11032, Invitrogen, USA) and Alexa Fluor 488 goat anti-rabbit antibody (A11008, Invitrogen) were used at a dilution of 1:100 in PBS as secondary antibodies. Cells after immunofluorescence staining were observed using a confocal laser scanning microscope (LSM800, Carl Zeiss Microscopy, Germany). Images were created by capturing 12 μm in the Z direction, to image the entire cell on the bottom of the media channel, and projecting the maximum fluorescence intensity onto the horizontal plane.

The localization of each protein was evaluated by analyzing acquired confocal microscopy images with the image processing software ImageJ (National Institutes of Health, USA). First, the relative area of VE-cadherin to the whole cell area was measured to evaluate the strength of adherent junctions. The outer and inner areas of VE-cadherin, A_{out} and A_{in}, were measured, and the following A^*_{cad} was calculated as the relative area of VE-cadherin [5,8,9].

$$A^*_{\mathrm{cad}} = \frac{A_{\mathrm{out}} - A_{\mathrm{in}}}{A_{\mathrm{out}}} \qquad (1)$$

In the evaluation of hypoxic sensing by the cells, the nuclear translocation rate of HIF-1α was evaluated. The nuclear image was binarized and multiplied with the HIF-1α image to generate an image of HIF-1α translocated into the nucleus. Then, the average fluorescence intensity in the nucleus $\bar{I}_{\mathrm{nucleus}}$ and the average fluorescence intensity of the whole image $\bar{I}_{\mathrm{whole}}$ were obtained to calculate the nuclear translocation rate of HIF-1α as $\bar{I}_{\mathrm{nucleus}}/\bar{I}_{\mathrm{whole}}$.

Each microscopy evaluation was performed using four devices. In addition, *z*-stacks of nine confocal microscopy images were acquired for each device and used for the analysis. For the significance test of the results between conditions, multiple comparisons were performed using one-way or two-way analysis of variance and Tukey's test, and it was determined that there was a significant difference when $P < 0.05$.

III. Results and Discussion

At the beginning of the observation of collective cell migration, the cell density was comparable between the different glucose conditions (Fig. 2). Hence, the glucose condition had no effect on cell viability during cell culture.

The migration speed of HUVECs over 5 h after supplying the gas mixture was measured by PIV analysis with the sequential phase-contrast microscopy images of the cells (Fig. 3). Variations in the migration speed in response to high glucose differed between hypoxia and normoxia. Under the normoxic condition N, high-glucose conditions HG and HG_{3days} significantly increased the migration speed of the cells compared to the normal glucose condition Ctrl. The migration speed gradually increased for 2 h by acute exposure to high glucose in the condition HG, approaching the value in the chronic high-glucose condition HG_{3days}, while it was almost constant in the condition HG_{3days}. On the other hand, under the hypoxic condition H1, both the control condition Ctrl and the chronic high-glucose condition HG_{3days} showed a peak migration speed around 2 h after the start of the experiment, which then decreased as time progressed. This transient variation agreed with the results of our previous study [9], in which nuclear translocation of HIF-1α in vascular endothelial cells also reached the maximum around 2 h after the exposure to hypoxia. In contrast, the acute high-glucose condition HG continued to decrease the migration speed over 5 h. Consequently, the effect of the oxygen concentration on migration of vascular endothelial cells was much more dominant than that of the glucose condition surrounding the cells.

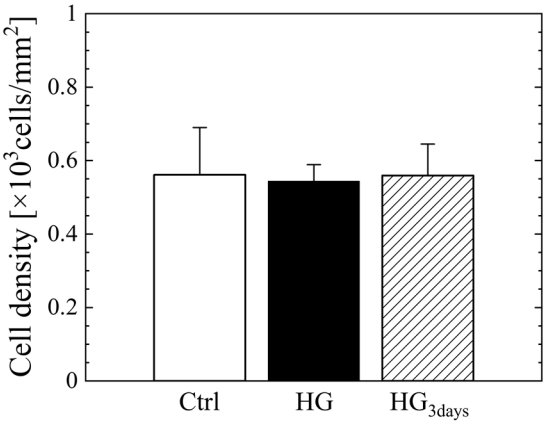

Figure 2. Cell density at the start of observation. The error bars show the standard deviation of independent experiments ($N = 8$)

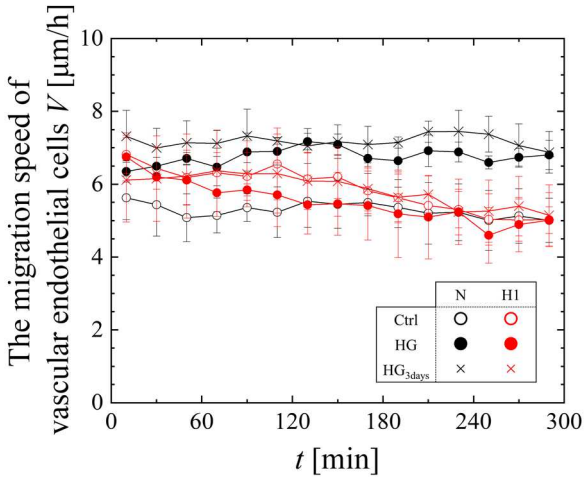

Figure 3. Variations in the migration speed of vascular endothelial cells. The error bars show the standard deviation of independent experiments ($N = 4$)

Representative microscopy images of VE-cadherin and HIF-1α after 5-h exposure to different conditions are shown in Fig. 4. VE-cadherin presented along the cell margin, and HIF-1α was translocated into the nuclei under the hypoxic condition H1 and the high-glucose conditions HG and HG_{3days}. The relative area A^*_{cad} of VE-cadherin slightly increased under hypoxia compared to normoxia (Fig. 5). The decrease in the migration speed by hypoxia in the present study could be attributed to the strengthening of cell-cell adhesion [5,8,9]. On the other hand, among the results obtained under the normoxic condition, changes in A^*_{cad} by high glucose were smaller than those by hypoxic exposure. Therefore, the increase in migration speed by high glucose seems not to depend on the cell-cell adhesion. When the cells were exposed to a high-glucose condition for 3 days (the condition HG_{3days}), A^*_{cad} decreased in both oxygen conditions, suggesting that intercellular adhesion was weakened by chronic exposure to high glucose.

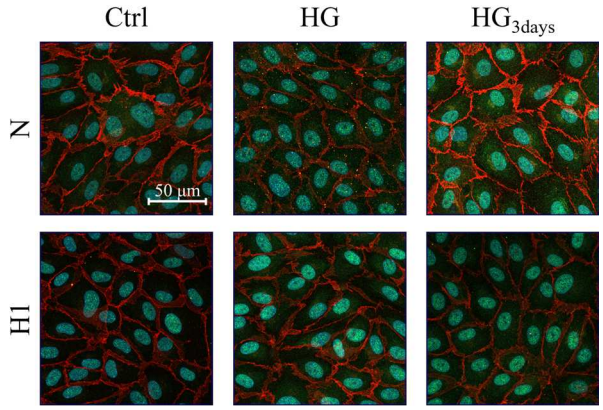

Figure 4. Representative microscopy images of VE-cadherin (red), HIF-1α (green), and nucleus (blue) in HUVECs.

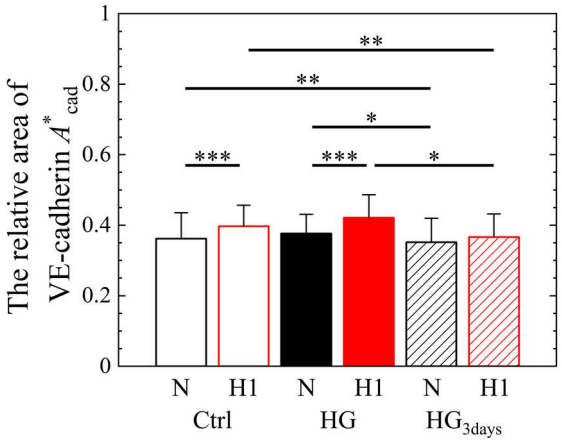

Figure 5. Relative area of VE-cadherin. Significant changes in relative area of VE-cadherin by glucose and oxygen conditions were assessed by two-way ANOVA, followed by Tukey's *post hoc* test for multiple comparisons. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. The error bars show the standard deviation of independent experiments ($N = 4$, 108 cells in total).

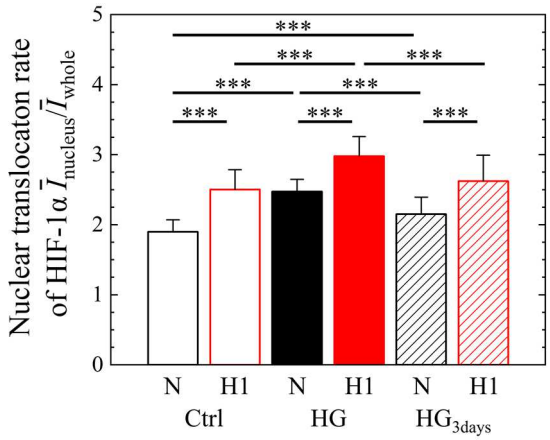

Figure. 6. The nuclear translocation rate of HIF-1α in HUVECs. Significant changes in the nuclear translocation rate of HIF-1α by glucose and oxygen conditions were assessed by two-way ANOVA followed by Tukey's *post hoc* test for multiple comparisons. $***P < 0.001$. The error bars show the standard deviation of independent experiments ($N = 4$, 36 images in total).

Furthermore, nuclear translocation of HIF-1α increased under a hypoxic condition in both glucose conditions (Fig. 6), indicating that the cells sensed the pericellular hypoxic condition irrespective of the glucose condition. Regardless of the glucose condition, nuclear translocation of HIF-1α was increased by 5-h exposure to hypoxia. However, the rate was smaller under the long-term exposure to high glucose than that under the acute exposure. A high-glucose condition reportedly increases mitochondrial oxygen consumption in cells and causes cellular hypoxia [10]. Our results suggested that HIF-1α is affected by cellular hypoxia due to the high-glucose condition, and this regulation is time-dependent. In addition, an excessive nuclear translocation of HIF-1α could associate with the decrease in migration speed of vascular endothelial cells, as shown previously [5]. Similarly, in the present study, the increase in migration speed induced by high-glucose exposure was not observed under the hypoxic condition because of suppression by the hypoxic response.

Cellular experiments using microfluidic devices have advantages in precise control of environmental factors and reducing the costs of cells and reagents. However, there are difficulties in quantitatively measuring proteins and gene expression levels due to the small sample volume. Such quantitative evaluations of cells using microfluidic devices require further improvement.

IV. Conclusions

Cellular experiments were performed using a microfluidic device to clarify the combined effects of glucose and oxygen on the behavior of vascular endothelial cells. The collective migration of vascular endothelial cells was dependent on both glucose and oxygen concentrations; however, the effect of the latter was more dominant. Cell-cell adhesion could be weakened with chronic exposure of cells to high glucose, though its involvement in the changes in glucose-induced migration was small. Further, high-glucose exposure seemed to induce intracellular hypoxia, and the hypoxic response of cells changed over time. The underlying mechanisms involved in vascular endothelial cell behavior, including the relationship between cell migration and HIF signal transduction and mitochondrial function in terms of energy metabolism on cell migration, will be further investigated in a future study.

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