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Normalization of high-flow or removal of flow cannot stop high-flow induced endothelial proliferation

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ABSTRACT

Endothelial cells (ECs) are activated in response to high-flow. Our previous studies using arteriovenous fistula (AVF) model have demonstrated that high-flow in blood vessels induces an early and rapid proliferation of ECs before arterial dilatation. Here, we investigated the proliferation of ECs, which had once been stimulated by high-flow loading, in a situation without the influence of high-flow. First, we induced high-flow in the rabbit common carotid artery by using AVF. Then, we removed the influence of high-flow by normalization of high-flow with the closure of AVF or by removal of flow itself with tissue isolation and organ culture or with cell culture of ECs, at the timing considered that ECs began to proliferate. Kinetics of ECs was investigated by a laser scanning confocal microscopy, phase-contrast microscopy and light microscopy using bromodeoxyuridine labeling method. We found that ECs, which had once been stimulated by high-flow, transiently proliferated even after normalization of high-flow or removal of flow. We assume that proliferation of ECs is promised when these cells start to proliferate after high-flow loading.

Arteries are remodeled in response to a change of blood flow or wall shear stress (2, 5). Endothelial cells (ECs) are considered to detect the wall shear stress on the surface of themselves and play an important role in arterial remodeling (4). Our previous studies have demonstrated that high-flow induces the proliferation of arterial ECs before arterial dilatation in dogs, rats and rabbits by using arterio-venous fistula (AVF) model (5–9, 11, 12). This proliferation of ECs is the most distinct and earliest morphological change (5, 9), and is an initial change of the remodeling. However, mechanisms of the proliferation are still uncertain. In our high-flow model performed so far (5, 9), high-flow was continuing during the whole experimental period. Therefore, it is not certain whether continuous high-flow is necessary during the whole period of the proliferation of ECs or only transient high-flow is required at the beginning of the proliferation of ECs. The purpose of this study is to investigate the proliferation of ECs, which have once been stimulated by high-flow loading, in a situation without the influence of high-flow. Here, we removed the influence of high-flow by 1) normalization of high-flow with AVF closure, 2) removal of flow with isolation and organ culture of the artery or 3) with *in vitro* culture of ECs immediately after the timing considered that ECs began to proliferate. Kinetics of ECs was investigated by bromodeoxyuridine (BrdU) labeling (1, 5, 8–10). We found that ECs once been stimulated by high-flow proliferated even after nor-

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malization or removal of high-flow.

MATERIALS AND METHODS

Our previous studies were demonstrated that the proliferation of ECs started at 1.5-day of high-flow and density of ECs was peaked at 3.0-day of high-flow, when high-flow was loaded in the rabbit common carotid arteries (CCAs) using AVF method (5, 9). Following this timetable, we planned high-flow loading for 1.5 days in the rabbit CCAs using AVF method. Then, we made flow normalization, flow removal, or *in vitro* culture of ECs and observed ECs for additional 1.5 days (at 3.0 days after creation of AVF) (Fig. 1).

We used adult Japanese white rabbits (weighing 3–4 kg; n = 39). All animal procedures were approved by the Animal Research Committee, Akita University School of Medicine and performed in compliance with the Guidelines for Animal Experimentation of Akita University School of Medicine.

1) High-flow experiments

1.1. Creation of high-flow

Animals were sedated with xylazine (4 mg/kg, intramuscular) and ketamine (25 mg/kg, intramuscular) and anesthetized with inhalation anesthesia of sevoflurane (1.5% in O_2/N_2O, 2/1 vol/vol). To induce high-flow, AVF was created between the left CCA and corresponding jugular vein with sterile techniques, following the procedures by Masuda *et al.* (5). Mean blood flow of the left CCA was measured by using an electromagnetic flow meter (Nihon Kohden Co., Tokyo, Japan) at 0.5 to 1.0 cm proximal to the thyroid artery branch before and after AVF and the increase of mean blood flow was confirmed. Animals without an AVF operation were used as control (n = 4).

1.2. BrdU administration

To label cells in synthesis phase (S-phase) of mitotic cycle, BrdU (50 mg/kg) dissolved in physiological saline solution was administrated by intraperitoneal injection at 1 h before finishing 1.5 days of AVF (1, 5, 8, 9, 10) in 15 animals. Because BrdU remains in blood flow only for about 10 min (3), this BrdU label method is regarded as pulse-labeling. Once BrdU is incorporated in DNA, it is kept remaining. Therefore it is considered that we can track BrdU-labeled ECs, which have once been in S-phase at

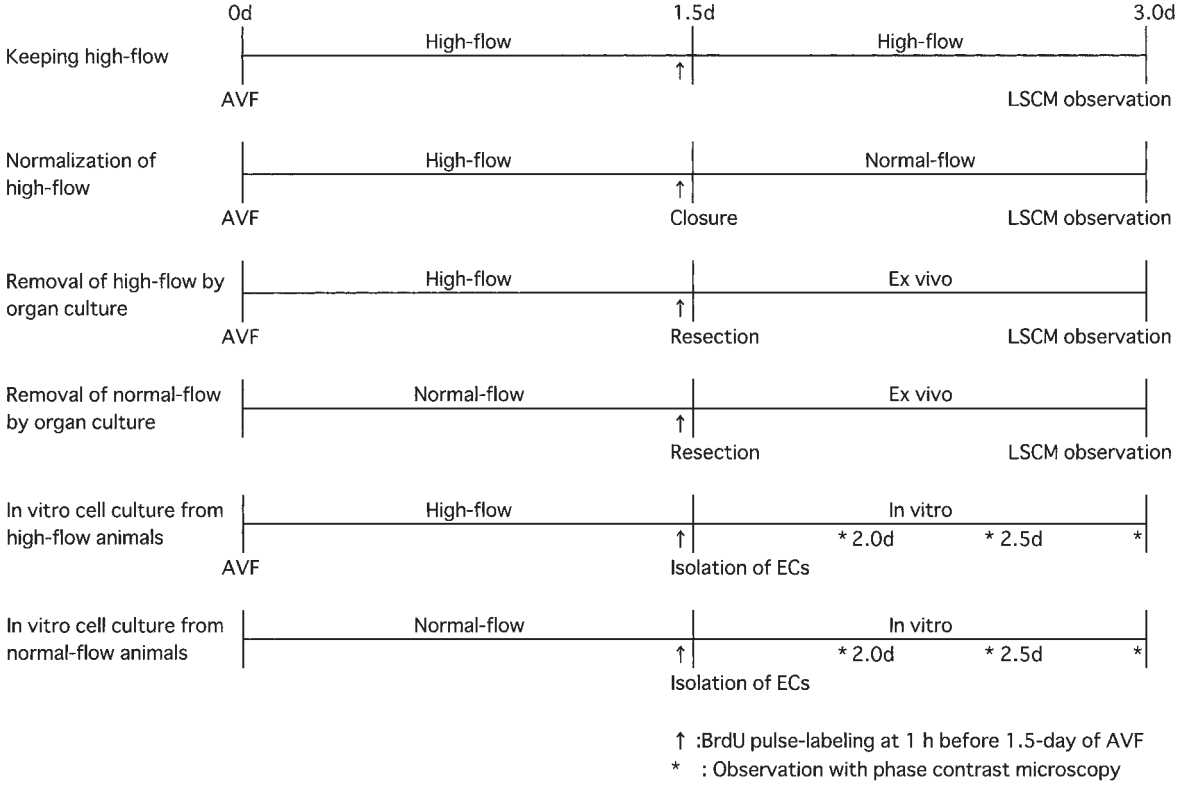

Fig. 1 Experimental protocols.

the pulse labeling time point, even after their mitotic division. Ten animals were euthanized at 1.5-day of AVF. Five animals were euthanized after 1.5 days tracking, namely at 3.0-day of AVF.

1.3. Terminal procedure
Before animals were euthanized, they were anesthetized similarly as described above (1.1.). After laparotomy, a catheter was inserted into the abdominal aorta at the portion of 2 to 3 cm distal to the renal arteries (1, 5, 8, 10). Animals were then euthanized by injection of an overdose of pentobarbital solution (100 mg/kg) through the aortic catheter. All animals were pressure perfusion fixed with 4% paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.4). Mean blood flow measurement was not performed at euthanasia, because the CCAs tend to cause spasm by the attachment of a flow meter probe and as a consequence *en face* microscopic observation of ECs becomes hard.

1.4. *En face* observation of ECs with a laser scanning confocal microscopy (LSCM)
The segments of the left CCAs, 0.5 cm in length, proximal to the thyroid artery branch were used for observation of ECs by a LSCM as previously described (1, 8, 10). After perfusion fixation, samples were frozen in PBS. After dissolving, they were incubated with 0.5% Triton X 100 in 2 N HCl for 30 min followed by washing with PBS and were blocked with goat serum (1 : 100) for 30 min at room temperature. Samples were then incubated with anti-BrdU mouse monoclonal antibody (Becton-Dickinson Immunocytometry Systems, San Jose, CA, USA) at 1 : 50 dilution in 0.1% BSA in PBS, which was detected with a fluorescein isothiocynate-conjugated anti-mouse IgG (Becton-Dickinson Immunocytometry Systems). The samples were then stained with propidium iodide for counter staining of all nuclei. After they were opened longitudinally, the whole wall of opened segments was dipped in 1, 4 Diazabicyclo [2.2.2.] - octane (Dako Cytomation Inc., Carpinteria, CA, USA) glycerin solution (50%) and mounted on the glass slide with the lumen side facing up, and covered with a cover slip for observation under LSCMs. The specimens were observed *en face* under LSCMs (LSM 410 and LSM 510 META, Carl Zeiss Co., Ltd., Oberkochen, Germany) using a HeNe laser (488 nm) and an Argon laser (530 nm). The HeNe laser stimulates fluorescein isothiocynate to emit green fluorescence, and the Argon laser stimulates propidium iodide for counter staining of all nuclei, emitting red fluorescence. ECs, which were not labeled with BrdU, were observed in red. ECs, which were labeled with BrdU, were observed in yellow to green, because they were emitted green and red fluorescence together. EC density and numbers of BrdU-labeled ECs were counted in each 320 × 320 μm image, and mean of five images were calculated.

2) Normalization of high-flow by AVF closure
At 1.5-day of AVF, animals (n = 4) were anesthetized similarly as described above (1.1.). Closure of AVF was performed by closing anastomosis with clipping. BrdU was administrated 1 h before the clipping as described above (1.2.). Normalization of blood flow of the left CCA was confirmed by using an electromagnetic flow meter. After 1.5 days of high-flow normalization, animals were euthanized as described above (1.3.). The segments of the left CCAs, 0.5 cm in length, proximal to the thyroid artery branch were used for *en face* observation of ECs by LSCMs as described above (1.4.). Mean blood flow measurement was not performed at euthanasia, because the CCAs tend to cause spasm by the attachment of a flow meter probe and as a consequence *en face* microscopic observation of ECs becomes hard.

3) Removal of high-flow by ex vivo organ culture
At 1.5-day of AVF, animals (n = 4) were anesthetized similarly as described above (1.1.) and the segments of the left CCAs, 0.5 cm in length, proximal to the thyroid artery branch were resected after BrdU administration 1 h before resection as described above (1.2.). Mean blood flow measurement was not performed at resection, because the CCAs tend to cause spasm by the attachment of a flow meter probe and as a consequence the resected CCAs contracted severely. The resected CCAs were cultured *ex vivo* without opening the lumen in EBM-2 (Cambrex BioScience Wakersvill Inc., Walkersville, MD, USA), including glutamine, penicillin, streptomycin, 2.0% fetal bovine serum at 37°C in an atmosphere of 5% CO_2 for 1.5 days. Samples from animals without an AVF operation and were cultured *ex vivo* for 1.5 days after BrdU administration 1 h before resection, and used as *ex vivo* control (n = 4). The resected CCAs including controls were fixed with 4% paraformaldehyde for 40 min after 1.5 days of *ex vivo* culture. They were processed for *en face* observation of ECs by LSCMs as described above (1.4.).

4) In vitro culture of ECs

At 1.5-day of AVF, animals (n = 4) were anesthetized similarly as described above (1.1.) and the segments of the left CCAs, 2.0 cm in length, were resected after BrdU administration 1 h before resection as described above (1.2.). Mean blood flow measurement was not performed at resection, because the CCAs tend to cause spasm by the attachment of a flow meter probe and as a consequence the resected CCAs contracted severely. The lumens of the resected CCAs were flushed by medium, EBM-2, including gentamycin and fungizone, to wash out the blood. Then, the vessels were treated with collagenase and incubated at 37°C in an atmosphere of 5% CO_2 for 30 min. After 30 min, the cells were isolated by centrifugation of collected flush medium at 800 rpm for 5 min. Isolated cells obtained by this procedure were in the range of $0.5 - 1.5 \times 10^4$ cells in each resected CCA. Some cells were clustered in various sizes consisting of up to about 60 cells.

The isolated cells were plated on type I collagen-coated glass cover slips (12 mm in diameter; Asahi Techno Glass, Chiba, Japan) and cultured in EBM-2 supplemented with glutamine, penicillin, streptomycin, 2.0% fetal bovine serum at 37°C in an atmosphere of 5% CO_2. The culture was maintained for 1.5 days (for observation of BrdU-labeled ECs) and 3.5 days (for evaluation of cell growth). The attached cells were observed with phase-contrast microscopy in every 12 h. In all the wells, many cells were attached as early as 0.5-day of cell culture. To evaluate cell growth, the numbers of cells were counted in three clusters in each animal. The individual clusters were observed in every 12 h and the growth ratio of each cluster (cell numbers of a cluster at each time point/cell numbers of the same cluster at 0.5-day of cell culture) was estimated. Each cell cluster can be followed till 3.5-day of cell culture based on the characteristic shape. To characterize the cultured cells, immunofluorescent stainings by CD31 and α smooth muscle actin were performed after the cells were fixed with 4% paraformaldehyde for 40 min. BrdU-labeled ECs were detected immunohistochemically by anti-BrdU mouse monoclonal antibody at 1.5-day of culture. Numbers of BrdU-labeled ECs were counted in five clusters, and mean of five clusters were calculated. Samples from animals without an AVF operation (n = 4) were cultured *in vitro* for 1.5 days after BrdU administration, and were used as control.

RESULTS

Blood flow

Mean blood flow of the left CCA prior AVF creation was 15–20 ml/min and it increased 40–60 ml/min immediately subsequent to AVF creation. These results was similar to that measured in our previous study (5). In normalization experiments, mean blood flow was 80–100 ml/min prior AVF closure at 1.5-day of AVF and 15–20 ml/min immediately subsequent to AVF closure, showing high-flow normalization procedure was succeeded.

High-flow experiments

At 1.5-day of high-flow, BrdU pulse-labeled ECs, which correspond to S-phase ECs, were often observed (157 ± 107 cells/mm^2; $6.3 \pm 4.3\%$, Fig. 2B), while in the usual physiologic state, very few BrdU pulse-labeled ECs (2 ± 1 cells/mm^2; $0.08 \pm 0.07\%$, Fig. 2A) were observed in the CCA. BrdU-labeled ECs appeared in 3214 ± 367 cells/mm^2 ($67.7 \pm 4.7\%$), when pulse-labeled animals at 1.5-day of AVF were tracked for 1.5 days (3.0-day of AVF) (Table 1 and Fig. 2C).

Normalization of high-flow

BrdU-labeled ECs appeared in 856 ± 264 cells/mm^2 ($28.3 \pm 7.1\%$), when pulse-labeled animals at 1.5-day of AVF were kept in a situation of flow normalization for additional 1.5 days (Table 1 and Fig. 2D).

Removal of high-flow by ex vivo organ culture

In animals, which were removed of high-flow after high-flow loading for 1.5 days and were maintained *ex vivo* for 1.5 days, ECs had tendency to be abraded from the internal elastic layer, and BrdU-labeled ECs were counted in the portion where ECs had not been abraded. BrdU-labeled ECs appeared in 1118 ± 265 cells/mm^2 ($35.4 \pm 8.4\%$) (Table 1 and Fig. 2E). In animals without AVF, which were maintained *ex vivo* for 1.5 days, a few BrdU-labeled ECs were observed (25 ± 21 cells/mm^2; $0.9 \pm 0.8\%$, Fig. 2F).

Isolation and culture in vitro

Isolated cells from the high-flow loading animals for 1.5 days were round in shape and about twice as large as erythrocyte. They sometimes gathered together forming clusters consisting of up to 60 cells. These cells were attached to type I collagen-coated cover slips within 0.5 days. At 0.5-day of culture, one growth layer of spindle-shaped cells grew radi-

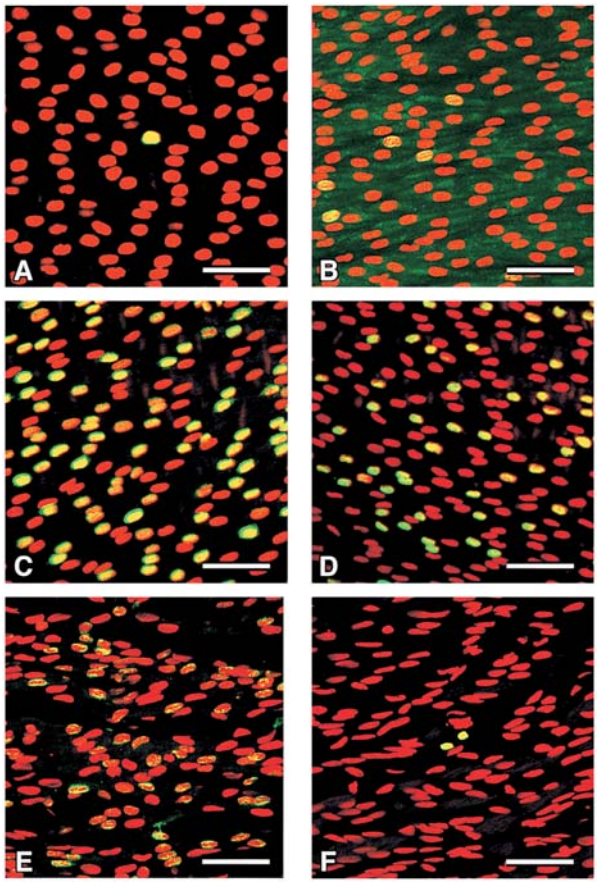

Fig. 2 LSCM images of BrdU-labeled ECs. All nuclei were counterstained red by propidium iodide. BrdU-incorporated nuclei are seen yellow-green.
(A) Normal-flow control (pulse-label). (B) 1.5-day of AVF (pulse-label). (C) Keeping high-flow (1.5 days after BrdU pulse-labeled at 1.5-day of AVF). (D) Normalization of high-flow (1.5 days flow-normalization after BrdU pulse-labeled at 1.5-day of AVF). (E) Removal of high-flow by organ culture (1.5 days flow-removal by *ex vivo* culture after BrdU pulse-labeled at 1.5-day of AVF). (F) Removal of normal-flow by organ culture (organ culture control). Bar, 50 μm.

ally from almost all the clusters (Fig. 3A). Single cell or twin cells occasionally appeared. At 1.0-day of culture, 2 to 3 growth layers of spindle-shaped cells appeared in almost all the clusters (Fig. 3B). There were no single cells, while twin cells or quadruplet cells clusters appeared. At 1.5-day of culture, spindle-shaped cells formed more than 3 growth layers to become slightly polygonal in the outermost growth layer (Fig. 3C). At 2.0-day of culture, spindle-shaped cells formed more than 4 growth layers and cells in the outer growth layer became polygonal. At 2.5-day of culture, more than 5 growth layers of cells grew (Fig. 3D). At 3.5-day of culture, number of cells was almost as many as that of 2.5-day of culture (Fig. 3E). Cells in the outermost growth layer became much polygonal and showed cobblestone appearance, however, cells in the innermost growth layer were still spindle-shaped. Number of cells in clusters increased from 0.5-day to 2.0-day of culture nearly to 14-fold as much as those of cells at 0.5-day of culture (Fig. 4). After 2.5-day of culture, number of cells in clusters remained almost unchanged.

Immunohistochemistry revealed that almost all the cultured cells were ECs expressing CD31 (Fig. 5B) and not expressing α-smooth muscle actin. At 1.5-day of culture, BrdU-labeled ECs appeared in $16.7 \pm 9.6\%$ (Table 1 and Fig. 5C). Isolated cells from normal-flow animals were in the range of $0.2 - 0.3 \times 10^4$ cells in each resected CCA. Isolated cells were single cell, twin cells, or forming clusters up to 10 cells. They were far fewer and smaller than those from the high-flow loading animals. Their cluster sizes and densities were almost unchanged, almost all of which were proved CD31 positive. There were no BrdU-labeled cells at 1.5-day of culture (Table 1). At 2.0-day of culture, we could not recognize these clusters of ECs.

DISCUSSION

Our previous study revealed that high-flow in blood vessels drove EC proliferation, where ECs started to enter the S-phase at 1.5-day after high-flow loading and progenies of the S-phase ECs at 1.5-day increased more than 10-fold at 3.0-day (9). We suggested that the S-phase ECs at 1.5-day proliferated continuously during additional 1.5 days.

However, it is not certain whether continuous high-flow is necessary during the whole period of the proliferation of ECs or only transient high-flow is required at the beginning of the proliferation, because during the period of the latter 1.5 days (from 1.5-day to 3.0-day of AVF) high-flow was kept high so far we have observed (5, 9). This study aims to investigate the proliferation of ECs, which have once been stimulated by high-flow loading, in a situation without the influence of high-flow.

In the present experiments, we could reconfirm that there was an increase of S-phase ECs, that is BrdU pulse-labeled ECs, at 1.5-day of AVF and a remarkable increase in the number of BrdU-labeled ECs at 3.0-day of AVF, which had been pulse-labeled at 1.5-day. If all the BrdU-labeled ECs at 3.0-day of AVF were the progenies of the BrdU pulse-labeled ECs (S-phase ECs) at 1.5-day, the S-phase ECs would increase more than 20-fold dur-

Table 1

	All ECs	BrdU-labled ECs	
	Density (cell/mm^2)	Number (cell/mm^2)	Labeling index (%)
Tracking of BrdU pulse-labeled animals *in vivo* and *ex vivo*			
Keeping high-flow (n = 5)	4810 ± 590	3214 ± 367*	67.7 ± 4.7*
Normalization of high-flow (n = 4)	2999 ± 300	856 ± 264†	28.3 ± 7.1†
Removal of high-flow by organ culture (n = 4)	3203 ± 438	1118 ± 265†	35.4 ± 8.4†
Removal of normal-flow by organ culture (n = 4)	3078 ± 756	25 ± 21	0.9 ± 0.8
In vitro cell culture of tracking of BrdU pulse-labeled ECs			
In vitro cell culture from high-flow animals (n = 4)			16.7±9.6
In vitro cell culture from normal-flow animals (n = 4)			0
BrdU pulse-labeling in controls, and 1.5-day of AVF			
Normal-flow (n = 4)	2698 ± 182	2 ± 1	0.08 ± 0.07
High-flow 1.5 days (n = 10)	2516 ± 285	157 ± 107*	6.3 ± 4.3*

Experimental protocols; keeping high-flow, normalization of high-flow, removal of high-flow by organ culture, removal of normal-flow by organ culture, *in vitro* cell culture from high-flow animals, *in vitro* cell culture from normal-flow animals, are explained in Fig. 1.

Data were shown as the mean ± SD.

*Significantly larger than normal-flow controls.

†Significantly larger than high-flow 1.5 days pulse labeling. ($P < 0.05$, student's t-test)

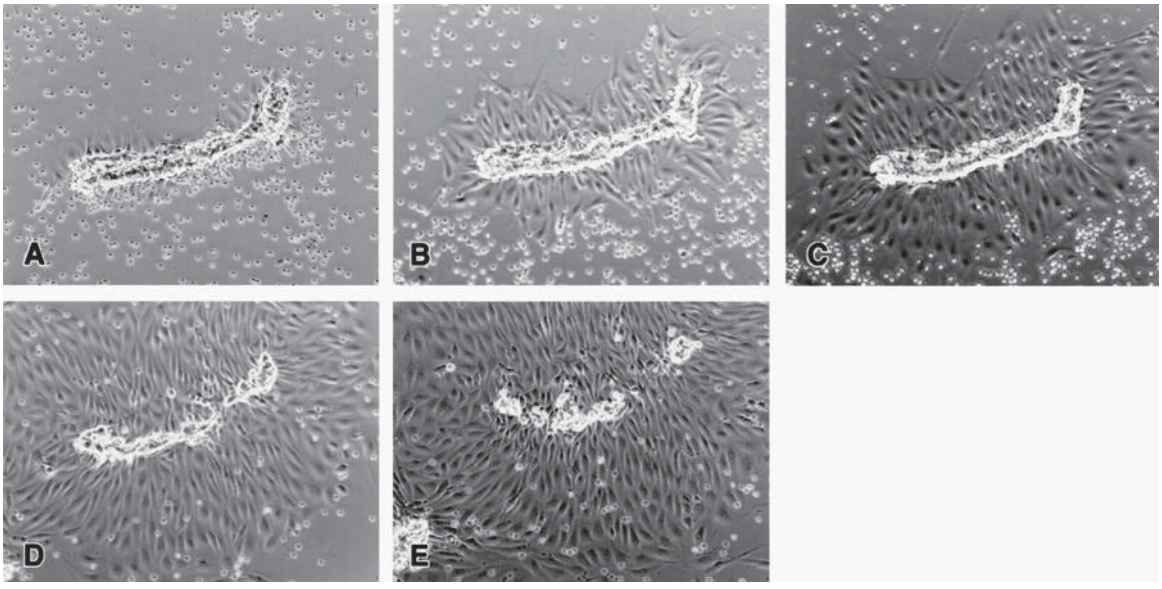

Fig. 3 Time course changes of a large cluster obtained from 1.5 days high-flow animals.
(A) 0.5-day, (B) 1.0-day, (C) 1.5-day, (D) 2.5-day, and (E) 3.5-day of culture. (Phase-contrast microscopy, original magnification, × 100)

ing this short period of 1.5 days. Although it is also considered that during 1.5-day to 3.0-day of AVF some BrdU pulse-labeled cells at 1.5-day of AVF in other parts of the body may migrate to become ECs, these S-phase ECs would divide several times during this short period of 1.5 days.

In normalization of high-flow experiments, BrdU-labeled ECs were 5.2-fold more than S-phase ECs of 1.5-day. They were one forth less than those in animals, which were pulse-labeled at 1.5-day of AVF and tracked until 3.0-day of AVF. If BrdU pulse-labeled ECs at 1.5-day divided twice, BrdU-labeled ECs would be 4-fold more than those of 1.5-day. Therefore it is probable that BrdU-labeled

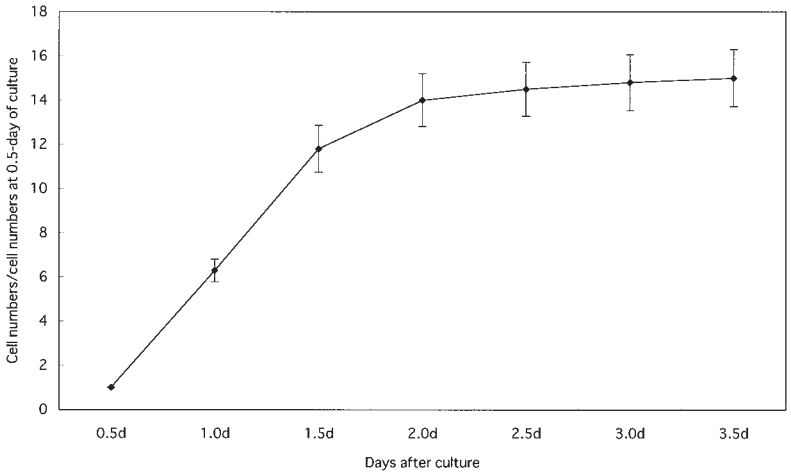

Fig. 4 The time course cell numbers of clusters obtained from 1.5 days high-flow animals. Ratio of cell numbers of a cluster at each time point/cell numbers of the same cluster at 0.5-day of cell culture were plotted according to the day after plating. Data are shown as the mean ± SE.

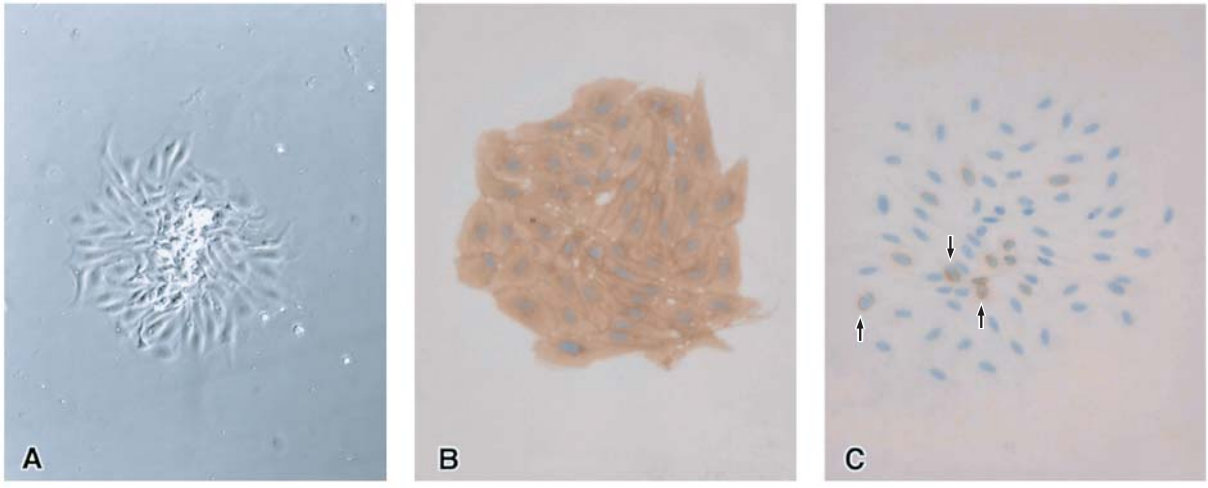

Fig. 5 Typical cluster of cultured ECs from 1.5 days high-flow animal at 1.5-day of culture.
(A) A typical medium sized cluster observed with phase-contrast microscopy, original magnification, × 100. (B) A typical medium sized cluster showed positive for CD31 immunocytochemistry. (Original magnification, × 200) (C) A typical medium sized cluster containing BrdU-positive cells. Arrows (BrdU-labeled ECs). (immunocytochemistry, original magnification, ×200).

ECs, S-phase ECs at 1.5-day of AVF, would divide more than once, although it is also considered that during 1.5-day to 3.0-day of flow-normalization period some BrdU pulse-labeled cells at 1.5-day of AVF in other parts of the body may migrate to become ECs.

In removal of high-flow by *ex vivo* experiments, BrdU-labeled ECs were about 7.1-fold more than those in animals, which were pulse-labeled at 1.5-day of AVF. They were one third as many as those in animals, which were pulse-labeled at 1.5-day of AVF and tracked until 3.0-day of AVF. However, in these experiments, because there appears no migration after removal, all the BrdU-labeled ECs 1.5 days after removal should be the progenies of the BrdU pulse-labeled ECs at 1.5-day of AVF. Therefore BrdU pulse-labeled ECs at 1.5-day of AVF would divide twice to three times during 1.5 days of *ex vivo*. From these *in vivo* and *ex vivo* experiments, we suspect that S-phase ECs at 1.5-day increase more than twice of mitotic divisions even though high-flow is normalized or flow is removal, while S-phase ECs at 1.5-day of AVF increased remarkably much more when flow is kept high.

However, these *in vivo* and *ex vivo* experiments lacked individual cell follow-up. That is to say that, in these experiments, relationship of BrdU pulse-labeled ECs at 1.5-day of AVF and tracked BrdU-labeled ECs after 1.5 days of high-flow or flow normalization or removal was only speculated, be-

cause these experiments used different animals at 1.5-day of AVF and after 1.5-day of flow alteration or removal.

To ensure the relationship of S-phase ECs at 1.5-day of AVF, S-phase ECs at 1.5-day of AVF after additional 1.5 days of AVF and those after 1.5-day of flow alteration or removal, we tried *in vitro* study. It revealed rapid increase (14-fold) of ECs from 0.5-day to 2.0-day of culture. After 2.5-day of culture, number of ECs remained unchanged. Therefore we consider that in culture from 1.5 days high-flow animals cultured ECs transiently proliferated by 3- to 4-divisions. Furthermore in the cultured proliferating ECs there were BrdU-labeled ECs at 1.5-day of culture, which were the progenies of the BrdU pulse-labeled ECs at 1.5-day of AVF, although their percentage was only 16%.

These results suggest that ECs, which had once been stimulated by high-flow loading, transiently proliferated even after normalization of high-flow or removal of flow. We assume two possibilities for the origin of these proliferating ECs. First possibility is that *in situ* ECs may gain character promised to proliferate during high-flow loading for 1.5 days. Second possibility is that the appearance of *de novo* ECs, which are promised to proliferate. In the present investigation, both possibilities are considered. Although further investigations are needed to confirm the origin of these proliferating cells, it seems that S-phase ECs at 1.5-day of high-flow are programmed to transiently proliferate through 3–4 mitotic divisions.

In conclusion, our study found that normalization of flow or removal of flow could not stop high-flow induced endothelial proliferation. ECs could proliferate in spite of normalization or removal of high-flow. These results suggest that specific ECs are involved in early stage of arterial remodeling and are promised to promote a proliferation program at the moment of beginning to proliferate.

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