Resident Endothelial Cells Surrounding Damaged Arterial Endothelium Reendothelialize the Lesion

Yoshiaki Itoh, Haruki Toriumi, Satoshi Yamada, Haruhiko Hoshino, Norihiro Suzuki

Objective—To evaluate endothelial repair processes in denuded pial vessels to clarify mechanisms for reconstructing endothelium (because endothelial repair of the cerebral artery after its damage is critical for the prevention of thrombosis, the maintenance of vascular tone, and the protection of the brain by the blood-brain barrier).

Methods and Results—Endothelial cells (ECs) in a 350-μm-long segment of the middle cerebral artery were damaged through a photochemical reaction. Tie2–green fluorescent protein transgenic mice were used for the identification of ECs. Six hours after the endothelial damage, ECs were detached from the luminal surface of the damaged artery, which was then covered with a platelet carpet. Within 24 hours, recovery of the denuded artery started at both edges, with EC elongation and migration. The repair rate was faster at the proximal edge than at the distal edge. Reendothelialization with EC proliferation peaked at 2 to 3 days and ended at 5 days, together with normalization of EC length, with no apparent involvement of foreign progenitor cells.

Conclusion—Our in vivo study demonstrated a stepwise reendothelialization process by resident ECs of the pial artery. The prevention of thrombosis, vasospasm, and treatment for blood-brain barrier dysfunction should be considered during the reendothelialization period. (Arterioscler Thromb Vasc Biol. 2010;30:1725-1732.)

Key Words: Tie2 ■ reendothelialization ■ pial artery ■ confocal laser microscopy ■ photochemical reaction

Because endothelial damage and subsequent exposure of subendothelial tissue in the cerebral artery quickly induces a luminal thrombus and disturbs cerebral blood flow, urgent endothelial repair is critical to minimize ischemic damage, especially after rupture of an atherosclerotic plaque, commonly observed at the carotid artery and the intracranial cerebral arteries. Reendothelialization is also essential to recover normal vascular tone controlled by vasodilatory mediators, such as nitric oxide released from the endothelium. Furthermore, loss of the endothelial tight junction results in breakdown of the blood-brain barrier, causing vasogenic edema. Therefore, restoration of endothelial function in the cerebral vessel is an urgent process for the brain.

Endothelial damage of the cerebral artery is a key mechanism involved in the pathophysiology of various brain diseases, including stroke, atherosclerosis, diabetes mellitus, dyslipidemia, hypertension, vasculitis, multiple sclerosis, radiation necrosis, and drug adverse effects. Some markers of endothelial damage are good indicators for the future incidence of cardiovascular events, including stroke. Carotid endarterectomy, a treatment for carotid stenosis, removes atheroma and endothelial cells (ECs), exposing subendothelial tissue. In these conditions, endothelial repair is one of the major targets of treatment.

Histological evaluation of reendothelialization and neointima formation after mechanical carotid denudation was previously reported. However, the dynamic and minute process of endothelial repair was not studied in detail, and mechanisms of endothelial repair are controversial. Although histological evaluation suggested better recovery with injection of “endothelial progenitor cells,” researchers reported that neither transplanted hematopoietic bone marrow cells nor spleen-derived mononuclear cells replace damaged ECs. However, they might, instead, facilitate reendothelialization via a paracrine mechanism after transmigration into brain parenchyma. Several novel methods were also reported to improve reendothelialization, including the use of cytokines and growth factors to stimulate resident endothelial growth.

Among various animal models of endothelial damage, rose bengal injection with mild laser illumination can selectively injure ECs and is regarded as the model that best resembles thrombosis in the setting of atherosclerosis. The resultant denuded segment is restricted to the area of laser illumination. Other methods of injuring endothelium include laser injury alone, topical application of chemicals (eg, ferric chloride), and mechanical damage, which may lack specificity in injured cell types, may not result in uniform denudation, or may require direct application of the toxic chemicals onto the brain surface.

To clarify repair processes of endothelial damage and to identify responsible cells for reconstructing endothelium, we
damaged ECs with the rose bengal method and evaluated time-lapse images of reendothelialization captured through the cranial window.

**Methods**

**Animals**

All procedures on animals were in accordance with the Animal Experimental Guideline of Keio University School of Medicine, Tokyo, Japan, and were approved by the Experimental Committee of Keio University.

Tie2 is a receptor of angiopoietins 1 and 2 and is expressed specifically in all ECs throughout development and in adults. Tie2-green fluorescent protein (GFP) transgenic mice were developed by Motoike et al., and fluorescent ECs can be specifically identified with fluorescent microscopy or confocal laser microscopy. Tie2-GFP mice were purchased from The Jackson Laboratory (STOCK Tg [TIE2GFP] 287Sato/J, Bar Harbor, Me). Male mice between the ages of 8 and 10 weeks were used for the experiment. To visualize blood vessel structures, CAG-EGFP mice were developed by injecting fragments containing the same age were obtained from Nippon SLC, Hamamatsu, Japan. The experimental Guideline of Keio University School of Medicine, Tokyo, Japan, and were approved by the Experimental Committee of Keio University.

**Cranial Window**

Animal preparation was conducted under 1.5% isoflurane inhalation anesthesia, with body temperature maintained at 37°C by a heat pad and with blood pressure monitored in a hind limb (MK-2000 ST; Muromachi Kikai Co, Ltd, Tokyo, Japan).

Under a surgical microscope, a metal bar was attached to the occipital bone with dental cement (Ionosit, DMG, Hamburg, Germany) to firmly hold the mouse head in an originally manufactured head holder during the following procedures. A 3-mm-diameter hole was drilled at 2.5 mm lateral and 2.5 mm posterior from the bregma above the left parietotemporal cortex without removing the dura. To protect the cortex from drying up and to keep the brain surface sterile during the long experiment, the trepanation was covered with a circular 140-μm-thick quartz glass window, sealed onto the bone with the dental cement. The mouse was then awakened, and 7 days were allowed for the recovery.

**Endothelial Damage**

Endothelial damage was induced via photochemical reaction of rose bengal (Sigma-Aldrich, Japan) to green laser light in wild mice (n=5), CAG-EGFP transgenic mice (n=5), and Tie2-GFP transgenic mice (n=8). By using 1.5% isoflurane anesthesia, a catheter was inserted into the tail vein. We used a 532-nm wavelength laser beam (Ventus 532; Laser Quantum, Cheshire, England) after adjusting its output to 150 μW with a photometer (model 8230; ADCMT, Tokyo, Japan) every time before laser illumination. The 350-μm-diameter laser beam, focused to the middle cerebral artery (MCA) through the cranial window, was projected for 150 seconds after intravenous rose bengal injection, 20 μg/g weight, in 50 μL of saline. The mouse was awakened again while being shielded from the room light for 6 hours to avoid an unintended photochemical reaction. In wild mice, rhodamine 6G (R6G), 20 μg/g weight, in 50 μL of saline was injected intravenously to label platelets just before the observation. Concomitantly stained white blood cells and pericytes were visually excluded by the size of the platelets on confocal laser microscopy.

**Time-Lapse Observation and Confocal Microscopy**

The injured cortical artery was observed through a cranial window with a confocal microscope equipped with an argon laser (λ=488 nm) (Melles Griot, Carlsbad, Calif), a confocal unit (CU-22; Yokogawa, Tokyo, Japan), a charge-coupled device camera (C4742-95; Hamamatsu Photonics, Hamamatsu, Japan), and an image analyzer (AquaCosmos, Hamamatsu Photonics). The observation of the reendothelialization process was periodical, and the mice were not under anesthesia between the observation. Reendothelialization rates were calculated separately at proximal and distal edges of the lesion by locating the endothelial tips at each point and dividing the distance by the time between the points. Regression of the edge was...
expressed as minus level. The length of ECs adjacent to the lesion was also measured and averaged.

**Immunohistochemistry and Statistics**

The brain of Tie2-GFP transgenic mice was removed after perfusion fixation with 4% paraformaldehyde (Wako, Osaka, Japan), cut into 15-μm-thick sections, and incubated with goat anti–von Willebrand factor (1:50, sc 8068; Santa Cruz, Santa Cruz, Calif), rabbit anti–platelet endothelial cell adhesion molecule (PECAM) antibody (prediluted, ab28365, Abcam, Cambridge, UK), or rabbit anti–α-smooth muscle actin followed by rabbit anti–goat IgG (heavy chain/light chain) tetramethylrhodamine B isothiocyanate conjugate (1:100, ZYMED 81–1614; Invitrogen, Carlsbad, Calif) or sheep anti–rabbit IgG (H+L) Texas red (1:50, ab6793, Abcam).

Endothelialization rates at each point were compared with the baseline level together with the comparison between the rate at proximal and distal edges using 2-way ANOVA, followed by multiple comparison. Cell length was analyzed in the same way.

**Results**

**Imaging of ECs**

Confocal laser microscopy revealed that pial arteries in CAG-EGFP mice were covered with long and narrow ECs (a in Figure 1A), whereas ECs of pial veins were round and short (v in Figure 1B). The identification of a pial artery or a vein was based on the connection of each vessel to the trunk of an MCA or major cortical vein under light microscopy (supplemental Figure; available online at http://atvb.ahajournals.org). This distinction was in complete accordance with the images of rose bengal injection, in which arteries, capillaries, and veins were visualized in turn. In a Tie2-GFP mouse, confocal laser microscopy revealed Tie2-positive cells covering all vessel walls, including arteries, veins, and capillaries (a, v, and c in Figure 1C and D), that can be confirmed by light microscopy. No Tie2-positive cells were found apart from the vessel walls. Serial confocal microscopy images of a branching MCA in Tie2-GFP mice showed densely assembled ECs in a single layer (supplemental video I; available online at http://atvb.ahajournals.org). A longitudinal section of the lateral vessel wall revealed that the EC layer in the artery was evenly thick with flat nuclei, whereas the thickness of the vein was uneven, with a moderate rise over round nuclei (Figure 1D). A 3D image of a pial artery and vein, which was reconstructed from confocal laser microscopy in the shape of a half cylinder and viewed from below, clearly demonstrates an EC monolayer lining the luminal surface of the pial vessels (supplemental video II).

**Immunohistochemistry in Tie2-GFP Transgenic Mice**

In sections for immunohistochemistry, Tie2-GFP–positive cells were found along the vessel wall in the cerebral parenchyma (Figure 2A and D). They were virtually all positive for von Willebrand factor (B, overlay C) and PECAM (E, overlay F) in capillaries (A–C) and a penetrating vessel (D–F). G, In addition, Tie2-GFP–positive cells (green) were surrounded by α-smooth muscle actin–positive cells (red) in a penetrating artery. The bar indicates 50 μm (A–C) or 10 μm (D–G).

**Endothelial Damage and Platelet Thrombus**

After starting endothelial damage by laser illumination and rose bengal injection, oscillatory vasomotion of the arterial...
wall followed by vasospasm was observed, suggesting endothelial dysfunction. During this photochemical reaction, platelet thrombus developed gradually on the injured vessel wall. Detachment of thrombus, followed by reproduction of fresh thrombus at the same site, was repeatedly observed during the injury. At the end of 150-second damage, the lumen of the injured artery was mostly filled with thrombus (semioclusion), blocking blood flow. These findings in wild mice after platelet labeling with R6G were clearly displayed in supplemental video III.

After 6 hours, light microscopy through a cranial window revealed that the injured artery was recanalized, with small thrombi still on some arterial walls. At 24 hours, virtually all arterial thrombi were already washed away with recanalized blood flow. The segment of damaged arteries still showed vasospasm, suggesting residual endothelial dysfunction. Live observation with confocal laser microscopy in CAG-EGFP mice (control in Figure 3A) revealed that ECs were lost in the injured arterial segment (bracket in Figure 3B); instead, a thin layer of platelets covered the denuded area. Higher-magnification images downstream (C) and upstream (D) of the lesion revealed that platelet coverage started and ended exactly at the edge of the remaining ECs (arrow). In a wild mouse, the injured segment of the arterial wall was brightly stained with R6G under a fluorescent microscope (E), which corresponded to a sheet of adhering platelets demonstrated with a confocal laser microscope (F). The size of the platelets on C, D, and F was 2 to 4 μm. The bar indicates 80 μm (A, B, and E) or 20 μm (C, D, and F).

**Figure 3.** A through F. Endothelial damage and platelet covering in CAG-EGFP mice (A–D) and wild mice with an R6G injection (E and F). A, Long and narrow endothelial cells covered the luminal surface of an intact pial artery. B, Twenty-four hours after endothelial damage by rose bengal and laser illumination, the denuded area (bracket) was covered with a thin layer of platelets. C and D, Higher-magnification images downstream (C) and upstream (D) of the lesion revealed that platelet coverage started and ended exactly at the edge of the remaining ECs (arrow). In a wild mouse, the injured segment of the arterial wall was brightly stained with R6G under a fluorescent microscope (E), which corresponded to a sheet of adhering platelets demonstrated with a confocal laser microscope (F). The size of the platelets on C, D, and F was 2 to 4 μm. The bar indicates 80 μm (A, B, and E) or 20 μm (C, D, and F).

Endothelial Repair

At 6 hours after a Tie2-GFP transgenic mouse was injected with rose bengal under laser illumination, only a trace amount of endothelial GFP fluorescence was detected in the damaged segment of the MCA and in the veins and capillaries within the laser-illuminated area, suggesting the detachment of most ECs (sky-blue arrowhead in Figure 4B and supplemental...
video VI) observed before the injury (Figure 4A). The fluorescence was completely lost at 24 hours in the damaged area, suggesting that all affected ECs were washed away (Figure 4C). In stark contrast to this loss of ECs in most segments of the lesion, reendothelialization had already started in the periphery at 24 hours (pink arrowhead in Figure 4C). Endothelialization continued and ended within 4 days on average (Figure 4D). The meeting point of distal and proximal reendothelialization ends consistently deviated distally from the center (Figure 4D).

Detailed and time-lapse observation of ECs surrounding the lesion with confocal laser microscopy at high magnification revealed that most ECs facing the border ended side by side, constituting the clear edge of the endothelium at 6 hours after the injury (Figure 5A). Some EC tips at the edges even seemed crumpled at the border (Figure 4B). Even thereafter, the extension of the EC tips to the damaged area was observed side by side, forming the clear edge of reendothelialization (supplemental video VII and supplemental video VIII). The growth of EC tips was generally slow in the distal borders, and regression of the EC tips was even observed at some distal edges (supplemental video VIII). The migration of the nucleus of the ECs was then observed (Figure 5B and supplemental videos VII and VIII). Some nuclei moved faster and passed other nuclei at times. Extending tips occasionally divided themselves, resulting in a fingerlike structure. The tip cell “fingers” may be parallel in the direction of blood flow (supplemental video VII) or curved and spreading, especially at a branching point (supplemental video VIII). Nuclear division, followed by the emergence of a fresh nucleus within a preexisting cell, was occasionally suggested on confocal laser microscope observation (cells 5, 6, and b in Figure 5B).

**Speed of Recovery in 3 Phases**

Based on the speed of recovery, 3 phases of recovery were noticed (Figure 6A). In the first phase (the cleanup phase), the growth rate was slow and even regression of the endothelial tip was observed, suggesting apoptotic cell death and detach-
The meeting point of 2 edges was statistically significantly higher than the distal reendothelialization rate. Indeed, the proximal reendothelialization rate was significantly higher than the distal reendothelialization rate. The direction of endothelial repair may affect the reendothelialization process.

**Figure 6.** A, Reendothelialization rates at proximal and distal edges of the lesion reached their peak at 2 and 3 days, respectively, after the endothelial damage (**P<0.01 and *P<0.05 vs baseline**) and returned to normal after 5 days. Recovery of the denuded artery was faster at the proximal edge than at the distal edge, especially in the early phase (†P<0.05). Regression of the endothelial tip at the distal edge of the lesion was even observed at 1 day after the damage. B, ECs at both edges became longer and reached their peak at 2 days after the injury (**P<0.01 and *P<0.05 vs cell length before the endothelial damage**). ECs at the proximal edge remained longer than those at the distal edge throughout the observation. The cell length returned to normal at 6 days after the injury.

**Discussion**

The repair of damaged endothelium of the cerebral vessel is an urgent reaction to secure cerebral blood flow and to protect the brain from a toxic substance in the blood. Although many diseases involve endothelial damage in pathophysiological features, and new treatments of such diseases are already targeted at the endothelial repair, little is known about how the damaged endothelium is reconstructed at the cellular level. To our knowledge, this is the first study to clearly demonstrate that resident ECs adjacent to the lesion mostly, if not exclusively, restore the endothelium by elongation, migration, and proliferation.

We used Tie2-GFP–transgenic mice to visualize ECs. The specificity of Tie2-GFP was evaluated in detail previously, which demonstrated that all Tie2-GFP–positive cells were positive for CD31. In the present study, we consistently observed that the luminal surface of virtually all vessel walls was lined with Tie2-GFP–positive cells. Conversely, we could not find any Tie2-GFP–positive cells apart from the lumen of the cerebral blood vessels, confirming the specificity and utility of Tie2-GFP as a marker of live ECs. However, any foreign cells that adhere to the vessel wall and differentiate to ECs may express Tie2-GFP. The possibility that some fresh ECs adjacent to the old ECs may be of foreign origin instead of resident cells after cell division cannot be excluded completely. The origin of new ECs found on the reendothelialized artery cannot be identified theoretically with this method.

However, in the present study, we demonstrated dynamically that elongation and migration of resident ECs started between 6 and 24 hours after the damage and continued until the completion of reendothelialization. The replacement of all resident ECs surrounding the lesion uniformly with progenitor-derived new ECs before 6 hours is unlikely. After 6 hours, we continuously observed reendothelialization, identifying each EC, and found elongation, migration, and proliferation of resident ECs. Continual observation revealed that tips of most elongating ECs moved exactly side by side to the uncovered area, forming the clear edge of reendothelialization. These responses were uniformly observed among ECs facing the lesion in all mice studied (n=8). No isolated segment of reendothelialization was found. Together with the previous results that neither bone marrow cells nor spleen- derived mononuclear cells were observed among the replaced ECs after transplantation, resident ECs are suggested to be the main source of EC replacement.

Discrepancy in the role of progenitor cells in reendothelialization among several reports may derive from the different animal models involved. The previous studies supporting the role of “endothelial progenitor cells” as a source of ECs used mechanical endothelial denudation that was associated with media damage, resulting in neointima formation, whereas the present study used a photochemical reaction that mainly damaged the endothelium. Also, the MCA was damaged in the present study, whereas the carotid artery was targeted in previous animal models involved. The previous studies supporting the role of “endothelial progenitor cells” as a source of ECs used mechanical endothelial denudation that was associated with media damage, resulting in neointima formation, whereas the present study used a photochemical reaction that mainly damaged the endothelium. The repair of damaged endothelium of the cerebral vessel is an urgent reaction to secure cerebral blood flow and to protect the brain from a toxic substance in the blood. Although many diseases involve endothelial damage in pathophysiological features, and new treatments of such diseases are already targeted at the endothelial repair, little is known about how the damaged endothelium is reconstructed at the cellular level. To our knowledge, this is the first study to clearly demonstrate that resident ECs adjacent to the lesion mostly, if not exclusively, restore the endothelium by elongation, migration, and proliferation.

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ous studies. The length of damage was rather short in the present study.

The rate of replacement was faster at the proximal edge than at the distal edge, suggesting that arterial shear stress in the same direction may facilitate migration and elongation of the ECs, whereas opposite shear stress may mitigate them. Because chemical mediators released from the lesion would be more readily conveyed to the distal vessel than to the proximal one, other mechanisms, such as contact activation between adhering platelets and ECs, may be involved in the induction of reendothelialization. The role of platelets in angiogenesis has indeed been widely reported, although the mechanisms are still unclear. Platelets contain numerous growth factors, including platelet-derived growth factors, vascular endothelial growth factor, epidermal growth factor, and fibroblast growth factor. Platelet-derived vascular endothelial growth factors were reported to exert trophic effects on cultured ECs. Thus, the previously mentioned angiogenic activators stored in platelets may locally promote the EC elongation, migration, and proliferation observed in this study.

We observed 3 phases of recovery. In the first phase, elongation and migration of the cells are the main mechanisms of reendothelialization. The rate of repair is slow. In the second phase, proliferation of the cells directly contributes to the coverage of the denuded area. The recovery rate reaches maximal. In the last phase, when the cells at both edges meet, they restore their original length by proliferation. The repair rate slows down to 0. This process resembles the stepwise induction of angiogenesis, during which sprouting of ECs, outgrowth of sprouts, and fusion of sprouts are sequentially observed. Fingerlike tip cells that we observed at the border of reendothelialization morphologically resemble outgrowing tip cells in angiogenesis and may be induced in a similar mechanism.

These results imply significant clinical relevance. In the present study, 350-μm-long MCAs were not covered with endothelium for 4 to 5 days after the insult. Clinically, endothelial damage in a segment this length or much longer can occur after the rupture of atherosclerosis or as a result of angioplasty or stenting in an intracranial cerebral artery. During this recovery period, the prevention of thrombosis and vasospasm and the protection of the blood-brain barrier should be considered. In the present study, relatively young male mice (aged 8 to 10 weeks) were enrolled. Although the length of the recovery period in human diseases cannot be speculated directly from the present study, any endothelial damage severe enough to occlude the intracranial artery may take 4 days or longer for reendothelialization.

The photochemical reaction of rose bengal with an illuminated laser was widely used in an animal model of thrombosis. Reactive oxygen species generated in the lumen are thought to be the main contributor of endothelial damage leading to thrombosis. Based on the resemblance of this process to atherosclerotic thrombosis, the method was used in many animal studies to investigate a thrombosis mechanism and to evaluate thrombosis treatment. The method was also used in a cerebral infarction model, in which the laser was illuminated to the whole parietal convexity. However, none of these reports focused on the repair process of the endothelium. Compared with the topical application of ferric chloride or direct laser injury, the photochemical method has the advantage that it can easily control the extent of vascular damage, limiting the damage to the endothelium and allowing the recirculation. The present study used this benefit and induced denudation of the affected artery, which allowed investigation of the reendothelialization process.

The present study suggested that facilitation of endothelial replacement is critical in the treatment of diseases involving endothelial damage. A number of recent articles focused on activation of “endothelial progenitor cells” or their extrinsic supply for treatment. As shown in the present study, such progenitor cells may not be involved directly as replacement of lost ECs. Instead, transplanted progenitor cells may facilitate migration and proliferation of resident ECs observed in the present study. New treatment to activate resident ECs should be explored more in the future.

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Disclosures

None.

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Supplemental Materials

Supplemental Figure Legend

Supplemental Fig 1. Pial arteries and veins observed through a cranial window in a control mouse. An artery or a vein is readily identified by tracing these vessels proximally to major vessels such as MCA, ACA or a large cortical vein shown in this figure. MCA: middle cerebral artery, ACA: anterior cerebral artery.

Online Video Legends

Online Video 1. Confocal Laser microscopy images of a branching middle cerebral artery in Tie2-GFP mouse. In this video, focus of the microscope starts at the deep level where the apparent diameter of the artery is maximum and is then moved to the top level of the artery. At the deepest level, longitudinal section of the lateral vessel wall displayed a single cell layer with flat nucleus. At the top level, several long and narrow ECs in tangential sections are tightly assembled side-by-side. (Scale 50 μm)

Online Video 2. Three-dimensional image of a pial artery and a vein viewed from below was reconstructed from confocal laser microscopic images. The rotating image clearly demonstrates the pial vessels in shape of a half cylinder with an EC monolayer lining the luminal surface of the vessel.

Online Video 3. The thrombus formation in the middle cerebral artery. In a wild
mouse, trace amount of rhodamine 6G was injected just before the endothelial damage by Rose Bengal and laser illumination through a cranial window. Laser-illuminated area is demonstrated as a large round dim area including cortical vessels, whereas platelet thrombus is shown as a highly bright aggregate inside the vessel. Adhesion of platelets to the injured site, aggregation of platelets, detachment of growing thrombus from the vascular bed and regrowth of thrombus are clearly visualized. At the end of 150 sec damage, branches are nearly occluded with thrombi. Images are shown 10 times faster.

**Online Video 4.** Confocal laser microscopy images of the distal edge of the lesion at 24 hours after endothelial damage in CAG-EGFP mouse. Still image is shown in Fig 3C. Focus of the microscope is moved toward the surface in the video. At the level of maximum diameter of the artery, platelets are rolling over the lateral wall of the proximal segment of the artery. No platelet thrombi are seen further to the center of the vessel where blood flow has already been restored. At the top level of the artery, the segment with massive platelet adhesion sharply ends at the edge of endothelial covering in the distal segment of the artery. Many platelets roll over the carpet of adhering platelets to distal direction and leave the vessel wall at the border. Diameter of the rolling platelets is 2 to 4 μm. Some of the rolling platelets extend their pseudopods, while others are discoid or spherical. As far as we observed, no platelets adhered directly to arterial EC. Images are replayed 7 times faster. (Scale 20 μm)
Online Video 5. Confocal laser microscopic images at 24 hours after endothelial damage in CAG-EGFP mouse, showing the upstream edge of the lesion. Focus of the microscope is moved to the surface in the video. Thin layer of adhering platelets starts at the end of the endothelium and some platelets start rolling over the vessel wall at the edge of endothelial covering. Images are shown 40 times faster. (Scale 20 μm)

Online Video 6. Confocal laser microscopic images at 6 hours after endothelial damage in Tie2-GFP mouse. The corresponding still image is shown in Fig 4B. Semi-occluded artery has already been recanalized and damaged ECs in the area of laser illumination (350 μm in diameter) are lost or barely seen as if there were no blood vessel between the intact vessels. Focus of the microscope is moved from deep level to the surface in the video. ECs in the lateral wall and, then, the top wall are demonstrated clearly on the intact vessel, whereas any ECs are barely seen in the damaged area. The edges of the lesion are sharply delineated, reflecting that the end of ECs facing the border is lining side-by-side. A pial vein is seen in the lower field of the images. (scale 100 μm)

Online Video 7. Elongation and migration of ECs at a proximal edge observed from 23 to 35 hours after the endothelial damage. Several elongated ECs are observed in a pial artery with their tips lining side-by-side forming the sharp edge of reendothelialization at 23 hours. The ECs extended their tips distally,
while some tips are divided themselves. Migration of the nuclei toward distal segment as well as emergence of fresh nuclei among the preexisting ECs are noticed during the observation. Finger-like structures are mostly parallel to vessel wall in this straight vessel in contrast to diverting form in a branching point shown in Online Video 8.

**Online Video 8.** Migration of EC and spreading of their finger-like structure at bifurcation of pial arteries at 23 to 35 hours after the endothelial damage. Several ECs on the edge show finger-like structure extending to the downstream denuded area at 23 hours. They migrate distally while stretching out their finger-like structure at the branching point.