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Impact of plasminogen on an in vitro wound healing model based on a perfusion cell culture system

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Abstract Vascular reorganization in wound healing is a complex process, which involves coagulation, endothelial cell proliferation and migration, basement membrane regeneration, and fibrinolysis. During this healing process, the hemostatic system and the angiogenic system are intimately interconnected. To elucidate the contribution of plasminogen in the process of wound healing, we have established a perfusion cell culture system. Using this novel cell culture system, we found that addition of plasminogen in the perfusion medium allowed the "scratch-wounded" endothelial cells to recover completely, while mini-plasminogen only affected the migration but not the proliferation of the endothelial cells. In the process of recovery with the addition of plasminogen, significant plasmin activity could only be detected when the growth of the endothelial cells have almost reached confluence. This finding indicates that wound healing is triggered and promoted during the absence of the proteolytic activity of plasmin. In addition, we could not detect any matrix metalloproteinase activity in the perfusion culture medium throughout the whole culture period. However, we did found that the circulating medium collected from the perfusion system at the early phase of the healing process has stimulatory activity on the growth of endothelial cells, but the proliferative activity decreased back to the basal level when the cells reached confluence. Thus, by using the perfusion cell culture system, we found that proliferation of endothelial cells is regulated by plasminogen and the wound healing process is controlled by a temporal interaction between the endothelial cells and plasminogen.

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Introduction

Wound healing is a complex process that involves cells, signaling molecules, and the extracellular matrices [1]. The cells participating in this process are the endothelial cells, which form a barrier between the vasculature and the blood flow, and the smooth muscle cells, which line the outer layer of the endothelium to strengthen the vessel wall. The signaling molecules produced by these cells as well as some of the components present in plasma exert a delicate balancing act on cell motility and proliferation to regulate the wound healing process. The extracellular matrix is also an important component of this process, because it provides the scaffolding and also acts as a reservoir for the growth factors.

In the process of wound healing, several factors contribute cooperatively to reorganize the damaged area. This process can be divided into three steps: an inflammatory step, a proliferative step, and a remodeling step. Moreover, the latter two steps can be grouped under the definition of angiogenesis, which involves the growth and development of new blood vessels from the pre-existing vasculature [2]. To counter act the effect of angiogenesis, the existence and ultimate identification of an angiogenesis inhibitor have also been reported [3–6]. Since the discovery of the angiogenesis inhibitor, designated as angiostatin [3], the biochemical and physiological nature of this plasminogen-derived peptide fragment has been extensively investigated by many laboratories [4–6]. In addition to angiostatin, a number of other plasminogen-derived peptide fragments have also been shown to inhibit angiogenesis in vivo and

proliferation of endothelial cells in vitro [7]. At present, many publications have appeared reporting on the regulatory effects of angiostatin on cancer metastasis [3–6], but as yet, the role of angiostatin as well as its precursor plasminogen on wound healing has not been extensively examined.

Angiostatin is generated from proteolysis of the fibrinolytic proenzyme, plasminogen, which is a 92-kDa glycoprotein, consisting of an amino-terminal preactivation peptide, five kringle domains, and a carboxyl-terminal serine protease catalytic domain. When angiostatin, which corresponds to the 1–4 kringles domains, is generated from plasminogen by the digestion of specific proteases, the amino-terminal preactivation peptide and mini-plasminogen should also be concomitantly produced [8]. Mini-plasminogen is the carboxyl-terminal portion of plasminogen starting from the fifth kringle domain, and it can be converted to a proteolytically active form, mini-plasmin. Although, at present, it is uncertain how angiostatin is generated under normal physiological conditions, one possibility is that it could be generated through the digestion by plasmin, followed by sulfhydryl rearrangement carried out by some reductants [9]. On the other hand, the cleavage between the Arg^{560} and Val^{561} bond of plasminogen by an urokinase-type plasminogen activator (uPA) or a tissue-type plasminogen activator (tPA) results in the generation of the proteolytically active plasmin with two chains, which are the kringles-containing A-chain and the catalytic B-chain, held together by disulfide bridges [10].

Plasmin is an important proteolytic enzyme in the fibrinolytic system and participates in the degradation of fibrin clots. Plasmin is also known to participate in inflammation, tumor metastasis, tissue remodeling, and angiogenesis. Recently, it has been demonstrated that plasmin plays an important role in tissue remodeling during wound healing and inflammation via proteolysis of the extracellular matrix components as well as via activation and/or liberation of growth factors from the matrices [11–15]. Using a genetically targeted animal model, Carmeliet et al. [16] reported that plasmin is involved in smooth muscle cell migration during the wound healing process. A significant role played by the plasminogen system in experimental wound healing in the lung, skin, and kidney was recently revealed by similar gene targeting studies in mice [17–19]. Furthermore, it has been shown that uPA and tPA are implicated in pericellular proteolysis during cell migration or tissue remodeling [20].

Even though a lot of research on the role of plasmin in the process of wound healing has been accumulated [11–20], studies on plasminogen itself and the temporal correlation between endothelial cell growth and plasminogen in wound healing have not been conducted extensively. The proliferative step in wound healing, consisting of endothelial cell growth and cell movement, occurs in between the coagulation and the fibrinolysis steps [21]. Once a blood vessel is damaged and followed by exposure of the subendothelial matrix, the coagulation system is activated. The damaged area is covered with a blood clot, consisting of fibrin and platelets. This triggers the proliferation of endothelial cells to replace the exposed surface. After the repair of the damaged area has been completed, the proliferation of endothelial cells is terminated and degradation of the fibrin clot is promoted. The plasminogen activator/plasminogen system plays a crucial role in this whole process [22, 23], as plasmin degrades fibrin clots, while angiostatin could act as an inhibitor of the proliferation of endothelial cells. Moreover, the timing of when plasminogen is converted to plasmin to exert its proteolytic activity has to be precisely regulated because a delayed conversion raises the risk of thrombus formation, whereas premature conversion may cause a hemorrhage. Therefore, elucidating the signaling molecules and pathways in the plasminogen activator/plasminogen system is going to provide a greater understanding of vascular development and may even lead to novel therapies for treatment of pathological conditions.

In the present study, we focused on the role of plasminogen in wound healing and adopted a perfusion culture system [24] of endothelial cells in order to investigate how plasminogen is involved in the endothelium wound repair, which is one important aspect of vessel reorganization. We used the culture of scratch-wounded confluent layer of bovine aortic endothelial cells as an in vitro model of endothelium wound repair. The confluent culture of endothelial cells on a cover glass was scratched off with a pipette tip and placed in the perfusion culture chamber. Under this culture condition with a continuous flowing medium supplemented with plasminogen, we observed the process of recovery of the endothelial cells on the cover glass and measured the time-dependent changes of protease activity and mitogenic activity in the circulating media collected from the perfusion chamber. We found that a bidirectional (reciprocal) interaction existed between the endothelial cells and fibrinolytic proteins in the process of wound repair.

Materials and methods

Materials

Human matrix metalloproteinase 2 (MMP-2) and human MMP-9 proenzymes were purchased from Alexis Biochemicals, Switzerland. Synthetic substrates for plasmin (Boc-Glu-Lys-Lys-MCA), plasminogen activators (Glt-Gly-Arg-MCA), and matrix metalloproteinases (MOCAc-

Pro-Leu-Gly-Leu-A_2pr(Dnp)-Ala-Arg-NH_2) were purchased from Peptide Institute, Inc., Japan. Urokinase-type plasminogen activator was obtained from American Diagnostica Inc., USA. Tissue-type plasminogen activator–inhibitor complex was purified from the conditioned medium of bovine endothelial cells according to the method of Sanzo et al. [25] with slight modifications. A cell-counting kit was purchased from Wako Pure Chemical, Japan. Porcine pancreatic elastase was from Roche Diagnostics, Germany. Chromatography media, lysine-Sepharose and Ni-chelating Sepharose, High S cation-exchange cartridge, and Cellulofine QAE-500 were purchased from GE Healthcare Bioscience, USA, Bio-Rad Laboratories, USA, and Kanto Chemical Co., Japan, respectively. The perfusion cell culture apparatus was purchased from Minucells and Minutissue GmbH, Germany. Cell culture media and the antibiotic-antimycotic solution were from Invitrogen, USA, fetal bovine serum (FBS) was from Biowest, France, and bovine serum (BS) was from Sigma, USA. Bovine aortic endothelial cells, generously provided by Dr. Soichi Kojima in RIKEN, Japan, were maintained in Eagle's minimum essential medium (EMEM), supplemented with antibiotic-antimycotic solution (final concentration: 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone) and 10% FBS ("maintenance medium").

Generation and purification of plasminogen, kringles 1–4 (angiostatin) and mini-plasminogen

Porcine Asp-plasminogen, the equivalent of Glu-plasminogen in human because porcine plasminogen has an Asp instead of a Glu at the N-terminal amino acid position, was purified from plasma by affinity chromatography on a lysine-Sepharose column, followed by anion-exchange column chromatography on Cellulofine QAE-500 as previously described [26]. Mini-plasminogen is the carboxyl-terminal portion of plasminogen starting from Gln^{450}, and it contains the last kringle domain, kringle 5, and the trypsin-like catalytic domain, B-chain. To generate mini-plasminogen and angiostatin, we first digested the purified porcine plasminogen with elastase at a protein/enzyme ratio of 200/1 in phosphate-buffered saline (PBS), pH 7.4. The digested mixture was then applied to a lysine-Sepharose column and the bound fraction, which contained the kringles 1–4 (angiostatin), was eluted from the column by a stepwise gradient of 6-aminohexanoic acid. The unbound fraction, which contained mainly mini-plasminogen and elastase, was dialyzed against 45 mM NaCl in 20 mM phosphate, pH 6.5, and then applied to a High S cation-exchange column. The mini-plasminogen retained on the column was separated from elastase by a stepwise gradient elution with NaCl. The purity and molecular size of mini-plasminogen were evaluated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and the N-terminal amino acid sequence of the purified mini-plasminogen was confirmed by Edman sequencing.

Apparatus and procedure for perfusion cell culture

The perfusion cell culture was performed in a closed system as described by Minuth [24] with slight modification. The system consisted of a cell culture chamber and a reservoir equipped with a ventilation filter, which were connected with tubing to form a closed loop. A peristaltic pump was installed in between the outlet of the chamber and the inlet of the reservoir to circulate the culture medium (Fig. 1). The cell culture chamber was placed on an incubation plate maintained at 37°C. Before proceeding with the perfusion cell culture, the cell culture cover glasses held by the minusheets in the culture chamber were placed one in each well of a 24-well tissue culture plate and endothelial cells were cultured on the cover glasses in the plate using the maintenance medium until the cells on the cover glasses reached confluence. The cover glasses were then washed with the "perfusion medium," consisting of EMEM, the antibiotic-antimycotic solution, 50 mM HEPES, which was used to maintain the pH of the system throughout the culture period, and 5% bovine serum (BS) instead of 10% FBS. In some experiments, BS was substituted with BS that had been depleted of plasminogen (ΔBS) by passing the serum through a lysine-Sepharose column [27]. The cover glasses with the attached cells were

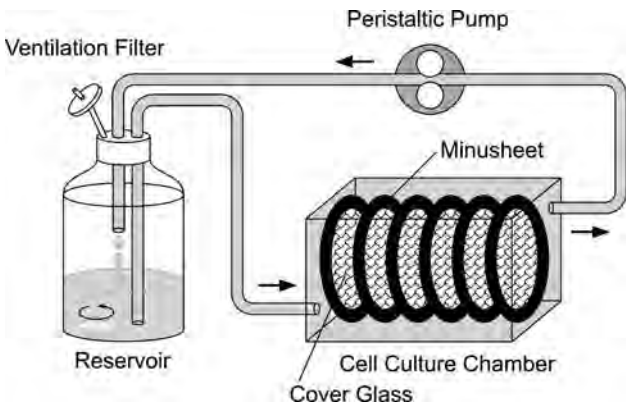

Fig. 1 A diagram illustrating the closed circulating perfusion cell culture system. Six cover glasses, each 13 mm in diameter, bearing the attached endothelial cells grown to confluence in a 24-well tissue culture plate were placed in the perfusion culture chamber and the chamber connected to the inlet and outlet tubing of a reservoir, which is equipped with a ventilation filter. After filling the system with the perfusion medium, a peristaltic pump was installed in between the chamber and the reservoir. The chamber was placed on an incubation plate to keep it at 37°C. The culturing was started by circulating the medium at a flow-rate of 1.5 ml/h while the reservoir was being gently stirred

taken out of the 24-well tissue culture plate and carefully set in the perfusion cell culture chamber, which could hold up to six cover glasses in line perpendicular to the direction of medium flow. The perfusion culture system was then filled with the perfusion medium and the perfusion culture was conducted for a total of 8 days with the same medium being circulated at a flow-rate of 1.5 ml/h. In those experiments in which a relatively large volume of the circulating perfusion medium was being collected for testing of its proliferative or proteolytic activity, the perfusion culture was performed in an open system. In this system two reservoirs, one feeding and the other collecting, and the cell culture chamber were connected in line and the system was perfused with the medium uni-directionally by using a pump installed in between the chamber and one of the reservoirs.

Assay for the recovery of the endothelial cells from scratch wounding under the perfusion culture conditions

The cover glasses with the attached confluent culture of endothelial cell were transferred to a 24-well tissue culture plate filled with the perfusion medium and the glasses were scratched with a pipette tip and then placed into the culture chamber. The cells were culture with the circulating perfusion medium, and at 24-h intervals the cover glasses were removed from the culture chamber and the cells examined under a Leica inverted microscope coupled to a CCD camera. After examination, the cover glasses were placed back into the culture chamber and the perfusion culture resumed. The whole process was carried out in a sterile tissue culture hood to maintain sterility. The extent of migration and proliferation of the endothelial cells, which represents the recovery from the damage, was quantified by using the Motic Images Plus Software and the results were expressed as a percentage of the recovered area. The percentage of the recovered area was calculated by subtracting the percentage of the nonrecovered area from 100% while the percentage of the nonrecovered area was calculated by dividing the nonrecovered area after 24, 48, and 72 h by the initial scratched area at time zero.

Assay for the proteolytic activities present in the circulating plasminogen-supplemented medium collected from the perfusion cell culture

The plasmin and plasminogen activator (PA) activities present in the circulating medium collected at 24-h intervals from the culture chamber were analyzed by using synthetic fluorescent substrates. To 0.99 ml of the collected medium was added 10 µl of the respective substrate solution adjusted to a concentration of 10 mM. The presence of plasmin and PA activities present in the medium would cause the liberation of the fluorescent compound, 7-amido-4-methyl-coumarin (AMC), and the increase in fluorescence intensity was determined by a JASCO FP-6200 Fluoro-spectrometer. The enzymatic activities present in the collected media were expressed as the relative amount of liberated AMC. The MMP activities present in the circulating medium were also determined by using the same procedure except that (7-methoxycoumarin-4-yl)-acetyl-Pro-Leu-Gly-OH (MOCAc) was used as the fluorescent reference compound.

Gelatin zymography assay of the gelatinase activity present in the circulating plasminogen-supplemented medium collected from the perfusion cell culture

The molecular masses of the proteins bearing the gelatinase activity present in the collected media were analyzed by gelatin zymography [28]. An SDS-polyacrylamide gel containing 0.1% gelatin in addition to the Laemmli ingredients [29] was prepared, and the collected medium samples were resolved on the gel under nonreducing conditions. The gel was then washed with a 2.5% Triton-X 100 solution for 45 min twice with gentle shaking to remove the SDS. The washed gel was placed in the development buffer solution, consisting of 150 mM NaCl in 20 mM Tris HCl, pH 7.4, and incubated for 48 h at 37°C. In some experiments, benzamidine (final concentration: 5 mM) or EDTA (final concentration: 5 mM) was added to the development buffer. After the incubation, the gel was washed and stained with Coomassie Brilliant Blue R-250. The protein bands bearing the gelatin proteolytic activity appeared as translucent bands in a blue-stained background.

Fibrin autography and reversed fibrin autography assay of the plasminogen activator and plasminogen activator inhibitor activities present in the circulating plasminogen-supplemented medium collected from the perfusion cell culture

Fibrinolytic activity present in the collected medium samples was examined by fibrin autography [30]. The samples mixed with SDS sample buffer were warmed at 60°C for 5 min and applied to SDS-PAGE. After completion of the electrophoresis, the gel was soaked in a 2.5% Triton X-100 solution for 45 min twice with gentle shaking to remove the SDS. Meanwhile, a fibrin-agar indicator gel was prepared according to the method as described by Levin and Loskutoff [30] with slight modification. Briefly, thrombin (0.5 U/ml) was added to a saline solution containing fibrinogen (6 mg/ml) and plasminogen (5 µg/ml), and rapidly mixed with an equal volume of 2% agar

solution at 48°C. The mixed solution was poured into a mold and let stand for 30 min to form the indicator gel. After the indicator gel had become firm, the soaked polyacrylamide gel was placed on top of the indicator gel and the two contacting gels incubated for 24 h at 37°C. The PA activities present in the polyacrylamide gel were identified as lysed zones on the indicator gel against an opaque background.

Plasminogen activator inhibitor (PAI) activity was examined by reversed fibrin autography [31]. The assay procedure was almost the same as that of the fibrin autography procedure except urokinase (0.05 U/ml) was also added to the fibrin indicator gel. The PAI activities present in the polyacrylamide gel were detected as clear white zones on the indicator gel.

Assay for the proliferative activity present in the circulating plasminogen-supplemented medium collected from the perfusion cell culture

The endothelial cell proliferation assay was carried out as follows. The cells cultured in the maintenance medium were washed with PBS and dispersed in a 0.05% solution of trypsin. The concentration of the cells suspended in the maintenance medium, supplemented with 5% instead of 10% FBS, was adjusted to 10,000 cells/ml after hemocytometer counting. The cells were then plated onto 96-well tissue culture plates at 0.1 ml/well and the plates were incubated for 24 h. The culture medium was then replaced by the same medium mixed with an equal volume of the sample medium collected from the perfusion culture. In this assay, the concentration of FBS in the culture medium was reduced to 2.5% in order to detect the proliferative activity present in the collected medium. However, the final serum concentration was always maintained at 5% by the addition of the sampled perfusion medium which contained 5% BS. After 72-h incubation, a cell-counting reagent from the Wako cell-counting kit was added to each well and the plate was further incubated for 2 h. The cell density in each well was then determined by measuring the absorbance at 405 nm.

Results

When bovine aortic endothelial cells were cultured on the cover glasses in the perfusion chamber with the circulating maintenance medium and the cover glasses removed from the chamber at 24-h intervals for examination under a microscope, the cells were found to proliferate at almost the same rate as if they were being cultured on the tissue culture dishes. The cells growing on each cover glass showed no difference in terms of proliferation. After the cells have grown to confluence on the cover glasses, they maintained a cobblestone-like shape, which is typical of endothelial cells, and became quiescent for at least one week (data not shown).

Effects of plasminogen, mini-plasminogen, and angiostatin on the growth of endothelial cells in the perfusion cell culture system

To define the role of plasminogen in the process of wound healing, we performed the perfusion culture of the endothelial cells recovering from scratch wounding in the presence or absence of plasminogen. The endothelial cells plated on cover glasses were grown to confluence in a 24-well tissue culture plate and six cover glasses with the attached endothelial cells were placed in the perfusion culture chamber with the cell-attached surface facing the inlet of the chamber and cultured for 24 h (denoted as day 0) with the circulating perfusion medium (Fig. 2a). The cover glasses were then removed from the chamber and the surface of each glass was scratched three times on different areas and washed with the serum-free medium. The cover glasses were then placed back into the culture chamber and the perfusion medium recirculated for another 24 h (denoted as day 1, see Fig. 2d). Subsequently, the cover glasses were removed from the chamber at 24-h intervals and examined for the extent of migration and proliferation of the endothelial cells, which represents the recovery from the damage.

When the culture was performed in the perfusion medium supplemented with plasminogen (20 μg/ml) starting from day 0, the endothelial cells steadily recovered from the scratch wounding. At 24 h after the cells were scratched (day 2; Fig. 2b), the cells have recovered to about 80% of confluence and at 48 h after the cells were scratched (day 3; Fig. 2c) the cells have reached confluence. During the recovery from the injury, we observed that the endothelial cells migrated to remote areas on the cover glasses and started to grow from there to form new colonies. This observation indicates that plasminogen stimulated both migration and proliferation of the endothelial cells. In contrast, when the cells were cultured only with the perfusion medium and without the supplemented plasminogen, the cells at the scratched edges migrated to unfilled areas very slowly on days 2 and 3 (Fig. 2e and f). It took the cells about 1 week to recover completely from the scratch wounding. Thus, the recovery speed of the endothelial cells from scratch wounding in the perfusion culture was approximately two times faster with the addition of plasminogen.

The same perfusion culture experiment was carried out with the addition of mini-plasminogen (10 μg/ml) instead of plasminogen to the perfusion medium. Under this

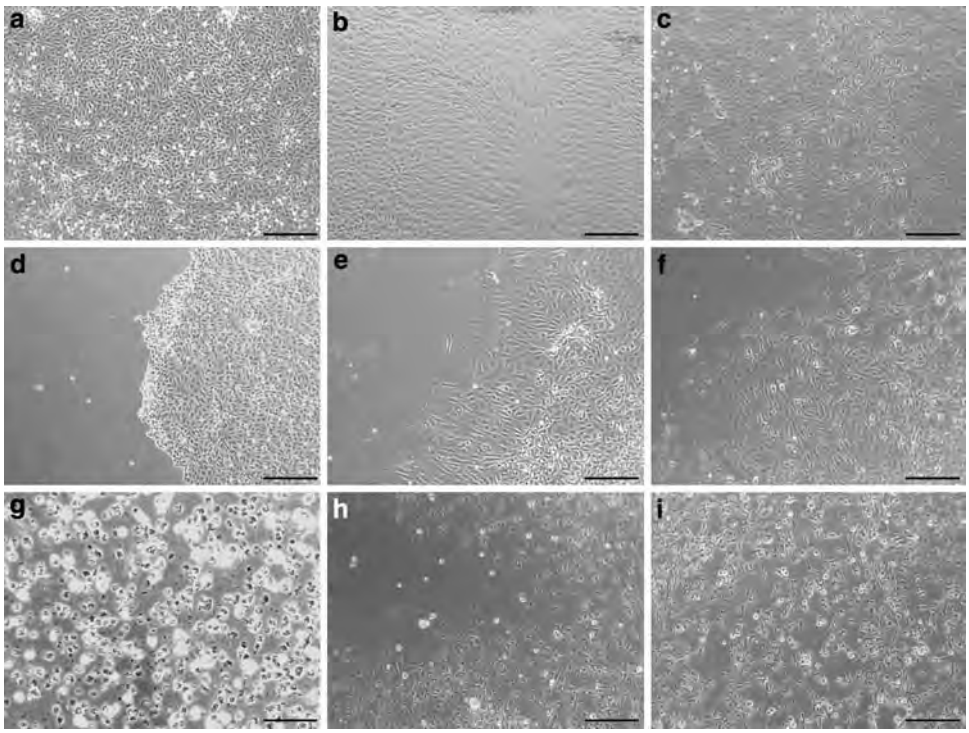

Fig. 2 Recovery of the endothelial cells cultured in control or various supplemented media after scratch wounding. A perfusion culture system was used to evaluate the effects of plasminogen and mini-plasminogen as well as angiostatin on the recovery of the endothelial cells after scratch wounding on the cover glasses for 7 days. In each experiment, the cover glasses were taken out of the chamber at 24-h intervals and examined for recovery of the cells from the wound. Panel **a**: confluent culture before scratching; panel **d**: scratched area at day 1; panels **b** and **c**: cells cultured in plasminogen-supplemented medium at day 2 and day 3; panels **e** and **f**: cells cultured in perfusion medium only at day 2 and day 3; panels **h** and **i**: cells cultured in mini-plasminogen-supplemented medium at days 2 and 4; panel **g**: cells cultured in kringles 1–4 (angiostatin)-supplemented medium at day 2. The bar in each figure denotes 100 μm

culture condition, mostly migration and with little proliferation of the endothelial cells were observed between day 2 (Fig. 2h) and day 4 (Fig. 2i), and the cells were growing in a scattered manner as well as maintaining a spindle-like shape. As a result, the cells ended up in a lattice-like pattern and did not reached confluence. To confirm the inhibitory activity of angiostatin on the growth of endothelial cells, the perfusion culture was performed in the presence of the plasminogen kringles 1–4 domains. Addition of kringles 1–4 (10 μg/ml) to the perfusion medium caused the endothelial cells to round up and detach from the cover glasses within 1 or 2 days after the perfusion culture was started (Fig. 2g).

Appearance of plasmin, plasminogen activator, and matrix metalloproteinase activities in response to recovery of the endothelial cells from scratch wounding

We then examined whether the accelerated recovery of the endothelial cells perfused with the plasminogen-supplemented medium was caused by the induction of plasmin and PA activities in the circulating medium. At the same times as the cells on the cover glasses were being removed from the culture chamber for examination, small aliquots of the circulating medium were collected and subjected to the measurement of plasmin and PA activities using synthetic fluorescent substrates. The circulating medium samples collected from the plasminogen-supplemented medium showed negligible amount of PA activity up to 4 days after the scratch wounding (Fig. 3; days 0–4 filled bars), but small amount of plasmin activity was detected in the first 2 days (Fig. 3; day 0–2 open bars). In days 3 and 4, when the endothelial cells have almost recovered to confluence from scratch wounding, the level of plasmin activity present in the collected media increased significantly. It is not clear how the added plasminogen was converted to plasmin in the early phase of the culture since the PA activity present in the same collected medium samples was extremely low. One possibility is that the sensitivity of the fluorescent substrate for measuring the PA activity used in this assay was too low to detect the activity present in the perfusion culture medium. Another possibility to explain the detection of plasmin activity could be the existence of other enzymes in the collected media that could cleave the same substrate used to detect the plasmin activity even though that substrate was designed with high

specificity for plasmin. Nevertheless, it seems that as the endothelial cells were growing to confluence in the first 3 days of the culture with the addition of plasminogen in the perfusion medium, these two protease activities did not contribute significantly to the proliferation of the endothelial cells because the PA activity was hardly detectable and the plasmin activity became significant only on day 3. Only when the cells were maintained in the confluent state for several more days did the PA activity increased to a noticeable level (Fig. 3, day 7 filled bar). As a result, activation of plasminogen to generate plasmin activity was drastically induced (Fig. 3, day 7 open bar). Since endothelial cells are also known to secrete several types of MMPs [11, 32–34], we also examined the presence of MMP activities in the collected culture medium samples. However, we could not detect any MMP activities using a fluorescent substrate for MMPs throughout the whole culture period (data not shown). The inability to detect MMP activities in the collected media might be caused by not using an appropriate substrate.

In an attempt to identify the nature of the proteases that were induced in the collected media, we examined their molecular masses by using gelatin zymography and fibrin autography. On the gel obtained from gelatin zymography of the circulating medium samples collected from day 0 to 5, the proteolytic activities appeared as bands with molecular masses of 92, 86, and 72 kDa (Fig. 4a). The band intensities at 92 and 72 kDa appeared to increase slightly from day 0 to 1, but remained at the same intensity levels thereafter. The proteolytic activities corresponding to the sharp bands at 92 and 72 kDa were diminished by the addition of EDTA (Fig. 4b, right panel: arrowhead), indicating that they could be matrix metalloproteinases MMP-9 and MMP-2, respectively, since these two metalloproteinases are specific for gelatin. The fuzzy band at 86 kDa was attenuated by the addition of the general serine protease inhibitor, benzamidine, in the development buffer (Fig. 4b, center panel: arrow). To identify which MMPs could exert the gelatin-digesting activities, we resolved pro-MMP-9 and pro-MMP-2 on the same gel and examined the migrating positions of these proteins. As shown in Fig. 4c, pro-MMP-9 migrated to the 92 kDa position and pro-MMP-2 migrated to the 72 kDa position (Fig. 4c, filled arrowhead), although an activated form of pro-MMP-2 was also detected (Fig. 4c, open arrowhead). These data indicated that the 92 kDa band and the 72 kDa band were the precursors of MMP-9 and MMP-2, respectively. Because the 86 kDa band was detected only in the culture medium collected from the plasminogen-supplemented medium but not from the control medium (Fig. 4b,

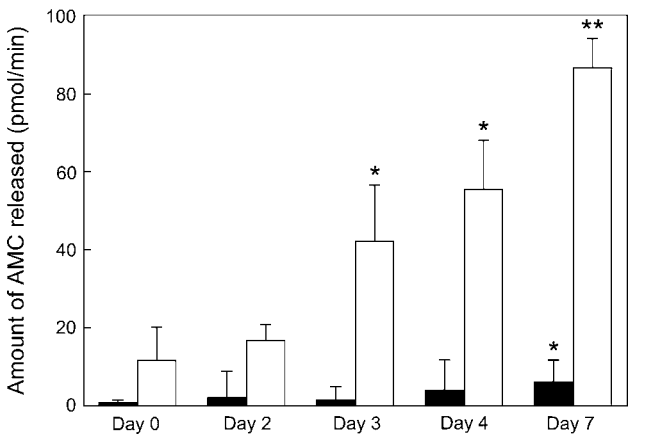

Fig. 3 Measurement of plasminogen activator and plasmin activities released into the culture medium by the perfusion cell culture. In the course of recovery of the endothelial cells after scratch wounding the cells were cultured in the perfusion system with the plasminogen-supplemented medium. At 24-h intervals a small aliquot of the circulating medium was removed at the same time as the cover glasses were being taken out of the chamber for examination. Plasminogen activator and plasmin activities present in each aliquot were measured using the fluorescent substrates. Filled bars denote plasminogen activator activity, whereas open bars denote plasmin activity. * $P \leq 0.05$, ** $P \leq 0.01$ versus day 0

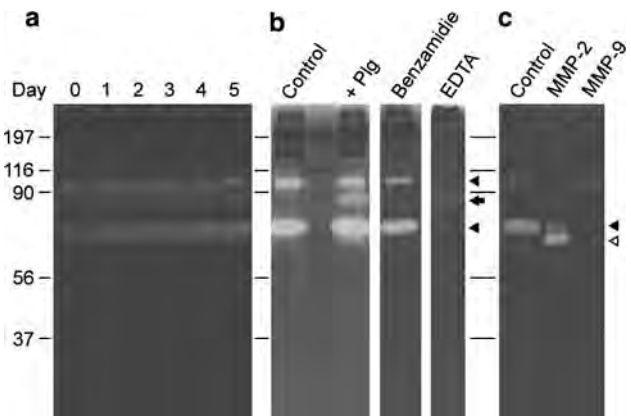

Fig. 4 Estimation of the molecular masses of the proteases released into the circulating medium by the cultured cells recovering from scratch wounding by gelatin zymography. **a** At 24-h intervals from days 0 to 5 of the perfusion culture with the plasminogen-supplemented medium, a small aliquot of the perfusion medium was collected and subjected to SDS-PAGE using a 0.1% gelatin-containing polyacrylamide gel. After washing, the gel was soaked in developing buffer for 48 h at 37°C. The gel was then stained with Coomassie Brilliant Blue R-250 for visualization. Molecular weight markers in kDa are shown on the left side. **b** A small aliquot of the perfusion medium collected from the control culture and the plasminogen-supplemented culture at day 2 were analyzed by gelatin zymography (*left panel*). The electrophoresized gels of the samples collected at day 2 from the plasminogen-supplemented culture were washed and soaked in the development buffer containing 5 mM benzamidine (*center panel*) or 5 mM EDTA (*right panel*), and then stained with Coomassie Brilliant Blue R-250 for visualization. **c** The collected medium sample from the control culture, and samples of pro-MMP-2 and pro-MMP-9 were analyzed by gelatin zymography. Note that the activated form of MMP-2 (*open arrowhead*) is also present in addition to pro-MMP-2 (*filled arrowhead*) in the electrophoresized sample

left panel), this band could represent the proteolytic activity of plasmin in which the plasminogen molecule had been activated but still interconnected by disulfide bridges between the cleaved A-chain and B-chains.

We next used fibrin autography and reversed fibrin autography to examine the PA and PAI activities present in the collected medium samples. Small amount of PA activity present in the collected medium samples at day 1 and day 3 from the plasminogen-supplemented culture was detected at a molecular mass of 100 kDa on the gel (Fig. 5a, filled arrowhead). In addition to the 100 kDa band, a faint PA activity band appeared at the 78 kDa position in the medium sample collected at day 3 (Fig. 5a, open arrowhead). The activity at the 78 kDa position seemed to increase slightly from day 3 to 5 and then remained at a constant level up to the end of the culture period while the activity at the 100 kDa position diminished gradually from day 3 to 5 and became undetectable thereafter (Fig 5a, day 3 and day 5 filled arrowhead). Since the migration positions of these activities coincided with that of the purified tPA-inhibitor complex and the dissociated form of tPA [25, 35, 36], and not with that of the uPA, these PA activities together with those found in Fig. 3 were assigned to the tissue-type plasminogen activator. Throughout the culture period, a constant level of plasminogen activator inhibitor-1 (PAI-1) activity was also detected as a band migrating at 52 kDa (Fig. 5b, arrowhead). Taken together, these results suggest that in the process of wound recovery several types of proteases and protease inhibitors are induced, which cooperatively regulate the proliferation of the endothelial cells.

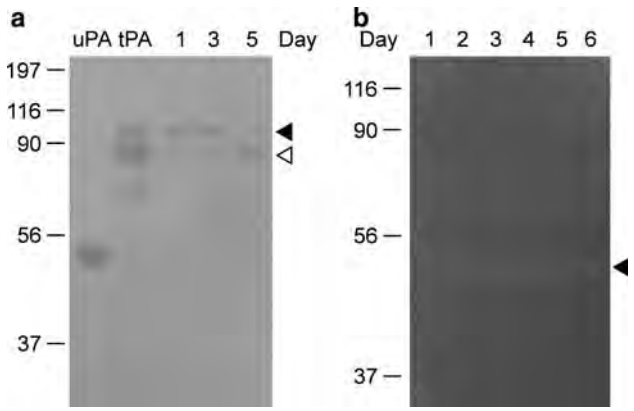

Fig. 5 Estimation of the molecular masses of the fibrinolytic and anti-fibrinolytic activities released into the circulating medium by the cultured cells recovering from scratch wounding by fibrin autography and reversed fibrin autography. **a** Fibrin autography. A fibrin indicator gel was prepared according to the method described by Levin and Losktoff [30] with a slight modification. Medium sample aliquots collected at days 1, 3, and 5 from the perfusion culture with the plasminogen-supplemented medium and samples of uPA and purified tPA-inhibitor complex were resolved on a 12% polyacrylamide gel. After washing, the sample gel was laid on top of a fibrin indicator gel. The two gels were then incubated for 24 h at 37°C. Fibrinolytic activities of the tPA-inhibitor complex (*filled arrowhead*) and the dissociated form of tPA (*open arrowhead*) plus uPA were identified as lysed zones against an opaque background. **b** Reversed fibrin autography. A fibrin indicator gel was prepared according to the method described by van Mourik et al. [31] with a slight modification. Medium sample aliquots collected at 24-h intervals from the perfusion culture with the plasminogen-supplemented medium were electrophoresized on a 12% polyacrylamide gel. After washing, the sample gel was placed on top of a fibrin indicator gel and incubated. The plasminogen activator inhibitor activities were detected as clear white zones (*arrow*) against a dark background

Detection of proliferative activity in the circulating perfusion medium

We next examined the changes in mitogenic activity present in the circulating plasminogen-supplemented medium. The perfusion culture medium was collected during the course of recovery from wounding at 24-h intervals and the collected media were evaluated by using the cell proliferation assay as described in "Materials and methods" section. As shown in Fig. 6, the media samples collected in the early phase of the perfusion culture in days 1 to 3, at a period when the cells were growing almost to confluence, showed proliferative activity with the day 3

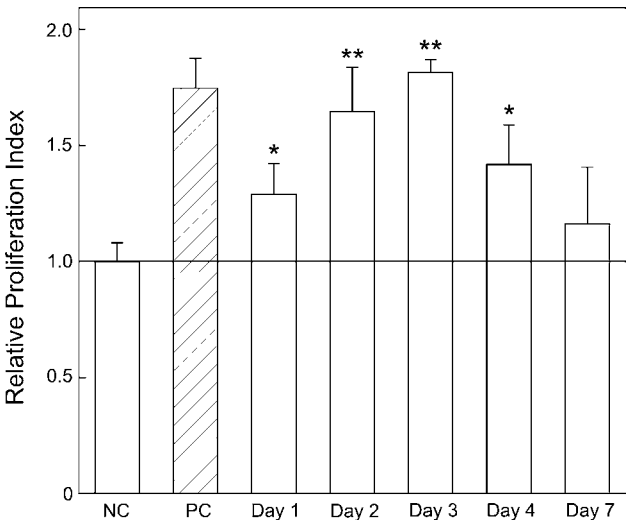

Fig. 6 Relative proliferative activities present in the circulating plasminogen-supplemented culture medium samples collected at 24-h intervals from the perfusion system. The proliferative activities were determined using endothelial cells. Briefly, endothelial cells were plated in 96-well microplates at 1000 cells/well and cultured with 5% instead of 10% FBS in the maintenance medium for 24 h to allow cell attachment. After washing, the cells were further cultured for three more days in the medium containing 2.5% FBS and 2.5% BS (negative control: NC), the same medium plus 10 ng/ml bFGF (positive control: PC), or the same medium mixed with an equal volume of the aliquots from the perfusion medium collected at 24-h intervals. The cell number at the end of the 3-day culture was measured using the cell-counting kit. * $P \leq 0.05$, ** $P \leq 0.01$ versus negative control

sample showing the highest. The measured activity on day 3 was as high as the positive control for the endothelial cell proliferation assay (Fig. 6; day 3 bar versus PC bar). In contrast, the proliferative activity started to decrease in the media collected in the later phase of the perfusion culture when the cells have reached complete confluence (Fig. 6; day 4). The mitogenic activity detected on day 7 was as low as the negative control (Fig. 6; day 7 bar versus NC bar) when the cells were kept at confluence. Thus, the circulating media collected from the actively growing cells contained strong mitogenic activity, whereas the media collected from the cells kept in a quiescent state showed little activity. These results indicate that actively growing endothelial cells release mitogenic factors to stimulate their own growth in an autocrine fashion or factors that interact with the constituents in the perfusion medium to stimulate their own growth.

Involvement of plasminogen in the recovery of endothelial cells from scratch wounding

To confirm the importance of plasminogen in the recovery from wounding, we next performed the same perfusion culture experiments with the medium consisted of plasminogen-depleted BS (ΔBS) and with the medium consisted of ΔBS plus 20 μg/ml of plasminogen. Substitution of BS with ΔBS in the perfusion culture medium had a profound effect on the recovery of the scratch-wounded endothelial cells. The wounded endothelial cells cultured in the ΔBS-containing medium did not show significant recovery at day 2 as shown in Fig. 7a, upper panel. By contrast, the wounded endothelial cells perfused with the ΔBS-containing medium that was supplemented with plasminogen had recovered steadily at day 2 (Fig. 7a, lower panel). Continual culturing of the scratch-wounded endothelial cells in the plasminogen-supplemented ΔBS-containing medium on days 2, 3, and 4 showed that the percentage of recovered areas reached 76%, 95%, and 99% of the originally scratched area, respectively (Fig. 7b, filled bars), whereas continual culturing of the wounded cells with the ΔBS-containing medium showed only 25%, 29%, and 36% recovery from scratch wounding on days 2, 3, and 4, respectively (Fig. 7b, open bars). These data indicate the importance of plasminogen on the recovery of the wounded endothelial cells.

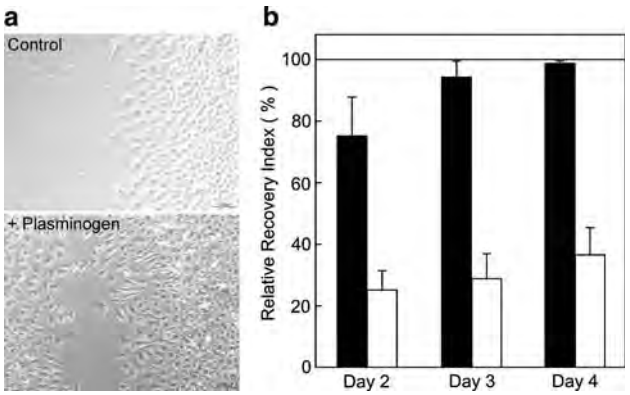

Fig. 7 Involvement of plasminogen on the recovery of the scratch-wounded endothelial cells cultured in the perfusion culture system. **a** Photographs showing the recovery status of the endothelial cells cultured with the ΔBS-containing medium (control) and plasminogen-supplemented ΔBS-containing medium (+Plasminogen). **b** The percentage recovery index of the cells at days 2, 3, and 4 cultured in the ΔBS-containing medium (open bars) and in the plasminogen-supplemented ΔBS-containing medium (*filled bars*). The percentages of the recovered areas were quantified from the photographs by using the Motic Images Plus Software

Discussion

Increasing evidence has shown that the fibrinolytic system contributes to many physiological processes, such as cell adhesion, cell migration, and intercellular signaling [37, 38]. These findings imply that plasminogen is a key molecule for tumor invasion in abnormal physiological conditions and for ovulation and wound healing in normal physiological conditions. In the present study, we have used a novel perfusion cell culture system to show that plasminogen but not plasmin was the key molecule for wound repair during the process of vascular reorganization. This system closely mimics the physiological environments for wound repair because the advantage of this system over a static culture system is that the factors, which have high affinity to the endothelial cells, are concentrated around the cells, whereas the factors, which have low affinity to the cells, are diffused away by the circulation. Using this system, we found that bovine aortic endothelial cells exhibited only a modest growth in the perfusion medium containing 5% BS (control culture). However, when plasminogen was added to the perfusion medium, the endothelial cells grew and reached confluence in less than half of the time it took for the control culture. Since we have purified plasminogen from BS before and, based on the yield, we estimated that the concentration of endogenous plasminogen present in the perfusion medium, which contained 5% BS, would have been approximately one-fiftieth of that of the plasminogen-supplemented medium. Thus, the amount of plasminogen present in the perfusion medium would be too low to exert a robust mitogenic effect. To exclude the possibility that the small amount of plasminogen present in the BS could exert some positive effects on the recovery of wounded endothelial cells, we carried out the perfusion culture in the medium containing 5% plasminogen-depleted bovine serum (ΔBS) in which plasminogen was removed by passing the BS

through a lysine-Sepharose column [27]. The extent of the recovery of the wounded cells cultured in the ΔBS perfusion medium was much lower than that of the normal 5% BS-containing perfusion medium. The cells cultured in the ΔBS medium did not recovered back to confluence even on day 7. By contrast, the cells cultured in the ΔBS medium plus the addition of exogenous plasminogen showed that the extent of the recovery from wounding was approximately the same as that of the plasminogen-supplemented normal BS-containing perfusion medium. These data strongly suggest that the recovery of the endothelial cells from scratch wounding requires the presence of the plasminogen molecule.

Several other studies have shown the involvement of plasmin in the process of wound healing [12–14]. Plasmin degrades the extracellular matrix components, activates growth factors, and affects cellular migration. These actions of plasmin were proposed to be responsible for promoting wound healing, whereas plasminogen itself, which has no proteolytic activity, was assumed to be just the precursor for plasmin. To determine how the proteolytic activity of plasmin contributes to the process of wound healing, we cultured endothelial cells in the perfusion culture system with the mini-plasminogen-supplemented medium. We used mini-plasminogen because plasmin present at the same concentration as plasminogen in the perfusion medium totally damaged the cultured cells and mini-plasminogen has moderate plasmin activity based on our own observation in the synthetic fluorescent substrate assay and gelatin zymography. Plasminogen also showed a weak proteolytic activity on the gelatin zymography possibly due to the conformational change caused by being exposed to SDS while it was being electrophoresized. Although the physiological role and the exact nature of mini-plasminogen were still unclear, several studies have shown that mini-plasminogen was formed in the course of plasminogen processing and might play some physiological roles. Leksa et al. [39] have demonstrated that mini-plasminogen was a high-affinity ligand for the mannose-6-phosphate receptor and was essential for the activation of TGF-β to induce endothelial cell apoptosis. Duboscq et al. [40] showed that mini-plasminogen was generated from plasminogen upon cleavage by leukocyte elastase but the cleavage product was unable to lyse fibrin clots even though it was activated to mini-plasmin. Because mini-plasminogen contains kringle 5, the molecule has been shown to bind to endothelial cells [41] and NG2 proteoglycan [42] through a binding site on kringle 5. Moreover, several laboratories [5, 8, 43, 44] had demonstrated that mini-plasminogen was concomitantly produced when angiostatin was generated through the digestion of plasminogen by MMP-3 and cathepsin D. And in the present study, we have found that addition of mini-plasminogen to the perfusion culture medium barely stimulated the proliferation of the endothelial cells but, rather, it stimulated their migration. These results could be explained by the presence of proteolytic activity in mini-plasminogen to detach the endothelial cells from the cover glass support so that they could migrate and this hypothesis is in accord with the previously reported observations, which demonstrated the involvement of plasmin in wound healing [11–15]. When angiostatin, the kringles 1–4 domains of plasminogen, was added to the perfusion medium, the cells did not grow to confluence as expected. Taken together, these results indicate that plasminogen and/or its degradation fragments affect positively and negatively the proliferation of endothelial cells in the process of recovery from scratch wounding.

Since plasminogen plays such a significant role in our perfusion culture system used to study wound repair, we attempted to identify the proteolytic activities responsible for activating and inhibiting plasminogen in this system. Endothelial cells have been shown to produce and secrete PAs, PAIs [45–47], and MMPs [11, 32–34] and the activation systems of these proteases/inhibitors are closely interconnected [48, 49]. Therefore, we examined whether the endothelial cells exerted any effects on the added plasminogen in the process of wound recovery through these proteolytic activities. From the results obtained from the digestion of the synthetic substrates, we found that PA activity was barely detectable in the circulating plasminogen-supplemented medium collected in the early phase of the healing (days 1–4). Only when the cells have reached confluence in the later phase of the culture did the PA activity became noticeable. Likewise, the results obtained from fibrin autography showed that the PA activity present in the circulating medium up to day 3 of the perfusion culture represented the tPA-inhibitor complex, which was the latent form and had no proteolytic activity [25, 35, 36], whereas this complex was replaced by the dissociated active form with a lower apparent molecular mass in the later phase of the culture. In contrast, a constant amount of PAI-1 activity appeared throughout the whole culture period. These findings help to explain the reason why very little PA activity was detected in the early phase of the perfusion culture but a significant PA activity could be detected in the medium collected on day 7 of the culture was due to the abundance of PAI-1 activity and the absence of the dissociated active form of tPA in the early recovery phase of the culture. While the endothelial cells secreted a constant amount of PAI-1 activity throughout the culture period, the inactive tPA-inhibitor complex was gradually replaced by the dissociated active form of tPA in a time-dependent manner and only the active form of tPA was present after day 5. This suggests that in the process of cell

proliferation in wound healing the endothelial cells control the activities of the fibrinolytic enzymes, depending on what recovery stages the endothelial cells are in. When the cells grew close to confluence on days 3 to 4, we could detect plasmin activity in the circulating medium even though there was no PA activity present in the same medium. Because plasminogen activation is regulated by a balance between PAs and PAIs within the extracellular environment [50, 51], either plasminogen is activated on the surface of the endothelial cells and the resulting plasmin released back to the medium or the plasminogen could be activated by other unknown PAs. In addition, it has been reported that PAI-1 is necessary for tumor invasion and vascularization through the activation of plasmin-mediated proteolysis [52, 53]. The reactivation of endothelial cells locally by scratch wounding could cause a spatially restricted activation of the fibrinolytic system and the activated system could turn into a temporally restricted regulator of the angiogenic system. Furthermore, several studies have reported that plasminogen was processed by some types of MMPs to generate several forms of angiogenesis inhibitors, including angiostatin [5, 8, 43]. However, we could not detect any MMP activity in the collected culture medium samples using a synthetic fluorescent substrate. Only by using the gelatin zymography assay could we detect two forms of MMP activities but the migration positions of these two forms corresponded to that of the proenzymes, one to pro-MMP-9 and the other to pro-MMP-2. Thus, the MMPs released into the culture medium were the inactive forms and it is inconceivable that these MMPs could contribute to the activation of plasminogen in the perfusion culture condition.

To examine how the proliferation of endothelial cells is dynamically regulated in the endothelial cell-plasminogen system, we assayed the proliferative activity present in the circulating medium collected at 24-h intervals. The media collected in the early phase of the culture (days 1–3), when the endothelial cells were growing extensively, showed stimulatory activity. However, the media collected in the later phase of the culture from day 4 onward, when the scratch wound had almost recovered, showed lower stimulatory activity. The medium collected at day 7, when the cells had become quiescent, showed that the stimulatory activity had reverted to the basal level. These observations show that there is a coordinated regulation of the mitogenic activities in the circulating perfusion medium, depending on the growing stages of the endothelial cells. The changes in mitogenic activity are controlled by factors secreted by the endothelial cells according to their growing status. When the growth of the cells has reached confluence, the cells will stop growing and the mitogenic activity in the medium disappears because there is no need for further growth.

Thus, by using the novel perfusion cell culture system, we have shown that proliferation of endothelial cells is regulated by plasminogen, which is a major participant of the fibrinolytic system, and this wound healing process is controlled by a temporal interaction between endothelial cells and plasminogen. The overall healing process is regulated by a sophisticated interplay between endothelial cells, growth factors, proteases, and various members of the extracellular matrix constituents. Even though further investigations are still required to unravel the details of the healing process, the present study showed for the first time that plasminogen plays a key role for wound repair in a proteolytic activity independent fashion. This finding could be taken as the first step to elucidate how the fibrinolytic system and the angiogenic system are dynamically interconnected in the process of vascular reorganization.

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