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Selenoprotein P as a diabetes-associated hepatokine that impairs angiogenesis by inducing VEGF resistance in vascular endothelial cells

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Abstract

Aims/hypothesis Impaired angiogenesis induced by vascular endothelial growth factor (VEGF) resistance is a hallmark of vascular complication in type 2 diabetes; however, its molecular mechanism is not fully understood. We have previously identified selenoprotein P (SeP, encoded by *SEPP1* gene in humans) as a liver-derived secretory protein that induces insulin resistance. Levels of serum SeP and hepatic expression of *SEPP1* are elevated in type 2 diabetes. Here, we investigated the effects of SeP on VEGF signaling and angiogenesis.

Methods We assessed the action of glucose on *Sepp1* expression in cultured hepatocytes. We examined actions of SeP on VEGF signaling and VEGF-induced angiogenesis in Human vascular endothelial cells (HUVECs). We assessed wound healing in mice with hepatic SeP over-expression or SeP deletion. The blood flow recovery after ischaemia was also examined by using hindlimb ischaemia model with *Sepp1* heterozygous knockout mice.

Results Treatment with glucose increased gene expression and transcriptional activity for *Sepp1* in H4IIEC hepatocytes. Physiological concentrations of SeP inhibited VEGF-stimulated cell proliferation, tubule formation and migration in HUVECs. SeP suppressed VEGF-induced reactive oxygen species (ROS) generation and phosphorylation of VEGFR2 and ERK 1/2 in HUVECs. Wound closure was impaired in the mice overexpressing *Sepp1*, whereas it was improved in $SeP^{-/-}$ mice. $SeP^{+/-}$ mice showed an increase of the blood flow recovery and vascular endothelial cells after hindlimb ischaemia.

Conclusions/interpretation The hepatokine SeP may be a novel therapeutic target for impaired angiogenesis in type 2 diabetes.

Keywords

angiogenesis, hepatokine, ROS, Selenoprotein P, VEGF

Abbreviation

HUVECs Human vascular endothelial cells

MAPK Mitogen-activated protein kinase

ROS reactive oxygen species

SeP selenoprotein P

VEGF vascular endothelial growth factor

Introduction

Type 2 diabetes is a chronic hyperglycemic condition that causes various vascular complications, including damage to small blood vessels resulting in retinopathy, nephropathy, and neuropathy, and damage to large blood vessels resulting in cardiovascular diseases. Earlier improved glycemic control is associated with reduced risk for cardiovascular disease in people with type 2 diabetes [1]. However,

more recent clinical trials have indicated that strict glycemic control does not necessarily prevent vascular complications [2]. Hence, beyond glycemic control, novel therapies to directly treat vascular disease are needed to improve the prognosis of people with type 2 diabetes.

Angiogenesis is a physiological process involving the growth of new blood vessels from pre-existing vascular structures and the subsequent formation of vascular network. A number of abnormalities associated with angiogenesis have been observed in people with type 2 diabetes [3], and impaired angiogenesis is linked to the development of various vascular complications in diabetes mellitus. Compared with control subjects without diabetes, people with type 2 diabetes show poor development of coronary collateral vessels on coronary angiography [4]. Moreover, a previous study using autopsied hearts reported that people with diabetes have significantly lower capillary densities in areas of myocardial infarction [5]. These reports suggest that the angiogenic response to infarction and/or ischaemia is inhibited at the levels of capillaries and small arterioles in type 2 diabetes. Inadequate vascular formation could attenuate perfusion recovery in response to ischaemia, thereby partially accounting for the poor clinical outcomes in type 2 diabetic patients with coronary heart disease or peripheral artery disease [6, 7]. In addition, insufficient angiogenesis is involved in abnormal wound healing and the development of diabetic skin ulcers [8].

Vascular endothelial growth factor (VEGF) is a major mediator of angiogenesis under physiological and pathophysiological conditions. VEGF binds and phosphorylates its receptors, leading to the activation of a variety of signaling cascades such as MAPK and Akt. Angiogenic gene therapy using

plasmids encoding VEGF has been attempted in patients with coronary or peripheral artery diseases [9]. However, diabetes mellitus people often show a poor response to therapeutic angiogenesis [10]. Therefore, VEGF resistance, a defect of VEGF-related signal transduction, has been postulated as a molecular basis for the dysregulated angiogenesis in diabetes mellitus [3, 11]. The molecular mechanisms underlying VEGF resistance in diabetes mellitus are not fully understood.

Selenoprotein P (SeP, encoded by the *SEPP1* gene in human and the *Sepp1* gene in mice) is a secretory protein produced primarily in the liver [12, 13]. It contains 10 selenocysteine residues and functions as a selenium supply protein [14]. We have previously reported that levels of serum SeP and hepatic gene expression of *SEPP1* are elevated in type 2 diabetes [15]. More recently, Yang et al. have reported that serum levels of SeP are increased in people with impaired glucose tolerance [16]. SeP impairs insulin signal transduction and induced dysregulation of glucose metabolism in skeletal muscle and liver, indicating that SeP functions as a type 2 diabetes-associated hepatokine that causes insulin resistance and hyperglycemia [15]. SeP has heparin-binding properties [17] and is associated with endothelial cells in rat tissues [18], suggesting that SeP exerts some actions on vascular endothelial cells. A previous paper showed that SeP has an anti-oxidative action in vascular endothelial cells by using in vitro experiments [19]. Nevertheless, it is unknown whether SeP plays a role in the angiogenic response.

We speculated that the liver-derived secretory protein SeP contributes to angiogenesis-associated vascular complications in type 2 diabetes by acting directly on vascular endothelial cells. In the current

study, we investigated the effects of SeP on angiogenesis in normal conditions, independently of diabetes, using purified SeP protein and *Sepp1*-deficient mice without the induction of diabetes.

Methods

Cell culture HUVECs were cultured in HuMedia EG2 (Kurabo, Osaka, Japan). H4-II-E-C3 cells were cultured in 10% FBS/DMEM (Gibco, Carlsbad, CA, USA) as previously described [20]. All cellular experiments were approved by the committee for cellular study at our institute.

Animals *Sepp1*-deletion mice were produced by homologous recombination with genomic DNA cloned from a Sv-129 P1 library [21]. All animal studies were approved by the committee for animal study at our institute. See ESM for further details.

Measurement of selenium Total selenium concentrations were determined by modification of Watkinson's method [22, 23]. See ESM for further details.

SEPP1 promoter assay The human *SEPP1* promoter region was cloned to a luciferase reporter vector, and luciferase activities were measured using the Dual Luciferase assay system (Promega) [20]. See ESM for further details.

Cell proliferation assay HUVECs were quantified using Cell Counting Kit-8 (Wako, Osaka, Japan). See ESM for further details.

Migration assay HUVECs were seeded in the upper chamber of polycarbonate filters, and the migrated cell number across the filter was counted. See ESM for further details.

Cell tubule formation assay HUVECs were seeded on plates coated with ECMatrix gel. Endothelial tubule formation was photographed under microscope. See ESM for further details.

Matrigel plug implantation assay This assay was performed using a Directed In Vivo Angiogenesis Assay Inhibition Kit (Trevigen Inc., Gaithersburg, MD, USA). See ESM for further details.

Western blot analysis HUVECs were pretreated with SeP for 24 hours. After 2 hours of starvation, HUVECs were stimulated with VEGF for 15 min. See ESM for further details.

RNA preparation and quantitative real-time Real-time PCR was performed on an ABI-Prism 7900HT (Applied Biosystems, Carlsbad, CA, USA). See ESM for further details.

ROS generation Intracellular ROS levels were measured using 2',7'-dichlorofluorescein diacetase (DCF) and quantified by Fluorescent plate reader (Fluoroskan Ascent FL, Yokohama, Japan). See ESM for further details.

Purification of SeP SeP was purified from human plasma using conventional chromatographic methods [14, 24]. See ESM for further details.

Preparation of human SEPP1 plasmid and overexpression of SeP in mice Human *SEPP1* expression plasmid was given from KAKETSUKEN (The Chemo-Sero-Therapeutic Research Institute, Tokyo, Japan). The plasmid was injected into the tail vein of mice. See ESM for further details.

Measurement of serum human SeP in mice injected with human SEPP1 plasmid Serum levels of human SeP were measured by enzyme-linked immunosorbent assays using two monoclonal antibodies [15, 25].

Mouse wound healing model Full-thickness wound was created, and the extent of wound closure was examined. See ESM for further details.

Hindlimb ischaemia model Mice underwent ligation and segmental resection of the left femoral vessel [26]. See ESM for further details.

Identification of $CD31^+$ vessels Antibody to CD31 was used for immunostaining. See ESM for further details.

Calculations and statistical analysis All data were analyzed using SPSS version 11.0 (Japanese Windows Edition; SPSS, Inc.) See ESM for further details.

Results

Glucose increases gene expression and transcriptional activity for SeP in cultured hepatocytes To confirm the elevation of SeP in the liver of people and animal models with type 2 diabetes [15], we examined the action of glucose on the production of SeP in H4-II-EC hepatocytes (Fig. 1). *Sepp1* mRNA expression was significantly increased by 25 mmol/l of glucose in a time-dependent manner (Fig. 1a). Additionally, *SEPP1* promoter activity as measured by luciferase activity was increased by 25 mmol/l of glucose compared with mannitol (Fig. 1b). These results are consistent with our previous findings showing that treatment with high glucose increases protein levels of SeP in mouse primary hepatocytes [15]. These results indicate that high concentrations of glucose increase the transcriptional activity of SeP in the cultured hepatocytes.

SeP impairs VEGF-induced angiogenesis in endothelial cells To assess the direct action of the liver-derived secretory protein SeP on vascular endothelial cells, we treated HUVECs with purified human SeP protein. HUVECs were treated with 5 or 10 μg/ml of purified human SeP protein, the levels of which correspond to serum levels of SeP in healthy subjects or people with type 2 diabetes [15]. In addition, we confirmed that levels of selenium were undetectable (less than 2.5 ng/ml) in all the culture media used for HUVECs. VEGF-induced proliferation of HUVECs was significantly suppressed by treatment with 10 μg/ml of SeP (Fig. 2a). Co-administration of buthionone sulfoximine (BSO), an inhibitor of glutathione synthesis, partly rescued the suppressive effect of SeP. Next, we examined the effects of SeP on VEGF-induced migration in HUVECs. VEGF promoted the migration of HUVECs into a polycarbonate filters. This migration was inhibited by the addition of SeP in a concentration-dependent manner (Fig. 2b-c). In the absence of VEGF, treatment with SeP did not affect the migration of HUVECs, suggesting that SeP modulates VEGF-dependent migration of endothelial cells. We further examined the effects of SeP on tubule formation in HUVECs. HUVECs cultured on Matrigel containing VEGF showed morphological tubule formation, with a lumen surrounded by endothelial cells adhering to one another (Fig. 2d). SeP inhibited tubule formation of HUVECs in a concentration-dependent manner (Fig. 2d-e). These *in vitro* results indicate that SeP at physiological concentrations impairs VEGF-dependent angiogenesis of vascular endothelial cells.

SeP reduces VEGF-stimulated formation of new vessels in Matrigel The role of SeP in angiogenesis *in vivo* was further determined by Matrigel plug implantation assay. Matrigel was mixed with VEGF in

the presence or absence of SeP protein and the plugs were implanted into the dorsal subcutaneous tissue of mice. Ten days later, angiogenesis inside Matrigel was quantified. SeP markedly inhibited VEGF-stimulated formation of new vessels in the Matrigel (Fig. 2f). These results further indicate that SeP impairs angiogenesis *in vivo*.

SeP impairs VEGF signal transduction in endothelial cells Next, we determined whether SeP affects VEGF signal transduction in endothelial cells. Pretreatment with SeP impaired VEGF-stimulated phosphorylation of VEGFR2 (Tyr1175) and ERK1/2 (Thr202/Tyr204) in HUVECs (Fig.3a-b). Co-administration of BSO partially rescued the inhibitory effect of SeP on VEGF signaling (Fig. 3a-b). The receptor of VEGF, VEGFR2 (KDR), mRNA expression in HUVECs was unaffected by treatment with purified human SeP protein (Fig. 3c). These results indicate that SeP at physiological concentrations impairs VEGF signal transduction in vascular endothelial cells.

SeP suppresses VEGF-induced acute generation of ROS in HUVECs To clarify the mechanism by which an anti-oxidative protein SeP impairs VEGF signaling, we assessed the action of SeP on the acute generation of reactive oxygen species (ROS) stimulated by VEGF. VEGF-induced ROS burst is reported to be required for the subsequent VEGF signal transduction [27]. Stimulation with 50 ng/ml of VEGF for 5 min significantly increased intracellular levels of ROS in HUVECs (Fig 3d). Pretreatment with SeP suppressed intracellular levels of ROS both with and without VEGF stimulation (Fig. 3d). These results suggest that SeP-induced VEGF resistance is associated with the reduction of ROS burst stimulated by VEGF.

SeP delays wound healing of skin in mice To clarify whether hepatic overexpression of SeP affects angiogenesis-related disorder *in vivo*, we used a hydrodynamic injection method to generate mice that overexpress human *SEPP1* mRNA in the liver. Levels of *SEPP1* gene expression in the liver and SeP protein in the blood were significantly elevated in these mice (Fig.4a-b), whereas serum levels of total selenium in wild-type and SeP-transgenic mice, which were 322.6 ng/ml and 331.0 ng/ml respectively, were not significantly different (Fig. 4c). We created excisional wounds (10 mm) in the dorsal skin of the mice and quantified the rate of wound healing. Wound closure was significantly impaired in the mice overexpressing *SEPP1* at 3, 5 and 7 days (Fig. 4d-e). In contrast, *Sepp1*$^{-/-}$ mice showed an improvement of the wound closure at 9 days compared with the wild-type animals (Fig. 4f-g). These results indicate that the hepatokine SeP delays the wound healing of the skin in mice.

Sepp1 heterozygous knockout mice show enhanced angiogenesis after hindlimb ischaemia To determine whether attenuation of SeP expression enhances angiogenesis *in vivo*, we generated hindlimb-ischaemia in *Sepp1*$^{+/-}$ mice. We previously reported that *Sepp1*-homozygous knockout mice exhibit enhancement of insulin signaling in skeletal muscle, whereas *Sepp1*-heterozygous knockout mice show marginal changes in insulin signaling [15]. Hence, we selected *Sepp1*-heterozygous knockout mice in the present study to assess the direct actions of SeP on the vascular system, independent of insulin signaling. 5 days after femoral artery ligation, *Sepp1*$^{+/-}$ mice showed a significant increase of blood flow compared with wild-type mice (Fig. 5a). This increase continued for 15 days after artery ligation (Fig. 5b). Consistent with these findings, histological examination showed

increased vessel density in the hindlimb musculature as determined by immunostaining with anti-CD31 antibody (Fig. 5c-d).

Discussion

The present study indicates that the liver-derived secretory protein SeP impairs angiogenesis both *in vitro* and *in vivo*. SeP directly attenuates VEGF signal transduction in vascular endothelial cells, resulting in suppression of VEGF-induced cell proliferation, migration, and tube formation. We reported previously that levels of both hepatic *SEPP1* mRNA and serum SeP protein are elevated in type 2 diabetes [15]. Taken together with our previous report, the present study suggests that hepatic overproduction of SeP may contribute to the onset of impaired angiogenesis in type 2 diabetes (Fig. 6).

The attenuated VEGF signal transduction, VEGF resistance, has been postulated as the molecular mechanism underlying the dysregulation of angiogenesis in people with type 2 diabetes [3, 11]. Waltenberger et al. reported that circulating monocytes show attenuation of VEGF-induced chemotaxis in people with diabetes mellitus [28] and that VEGF-stimulated phosphorylation of downstream molecules is reduced in monocytes from patients with type 2 diabetes [29]. In addition, Sasso et al. found impaired VEGF signaling in the myocardium of type 2 diabetic patients with coronary heart disease [30], suggesting that diabetes induces VEGF resistance in not only monocytes but also other types of cells such as cardiomyocytes and endothelial cells. However, the molecular mechanisms by which VEGF resistance arises in diabetes mellitus have not been elucidated. The

results of the present study suggest a novel molecular pathology of type 2 diabetes; elevation of circulating SeP induces VEGF resistance in vascular endothelial cells.

The liver is the production site of various secretory proteins. Recent work in our laboratory has indicated that genes encoding secretory proteins are abundantly expressed in the liver in type 2 diabetes [31]. Moreover, genes encoding angiogenic factors, fibrogenic factors, and redox-associated factors are differentially expressed in the liver in type 2 diabetes, possibly contributing to the pathophysiology and clinical manifestations of this disease [32, 33]. The present study sheds light on a previously under-explored function of the liver; the liver may participate in the regulation of systemic angiogenesis by altering the production of angiogenesis-associated hepatokines such as SeP.

Our observation that SeP impairs angiogenic processes is noteworthy in the context of experimental data suggesting that SeP plays a role in the anti-oxidative defense system [13]. In fact, we have shown previously that SeP increases the activity of glutathione peroxidase 1 (GPX1), a representative anti-oxidative enzyme that requires selenium for its enzymatic action, in Jurkat E6-1 cells, a human T-cell leukemia cell line [14]. SeP-induced activation of GPX1 was also demonstrated in endothelial cells [19]. Accumulating evidence indicates that reactive oxygen species (ROS) stimulate angiogenic response in order to initiate the tissue repair process in ischaemia-reperfusion lesions [34]. Among the growth factors involved in angiogenesis, VEGF plays a role in a ROS-dependent signal transduction system [27]. VEGF binding to VEGFR2 stimulates NADPH oxidase in endothelial cells, resulting in acute generation of ROS such as hydrogen peroxide. This ROS burst oxidizes and inactivates protein

tyrosine phosphatases, which negatively regulate VEGF signaling, and thereby promote VEGFR2 phosphorylation and the subsequent signaling cascade [27].

In combination with these previous reports, the present data suggest that SeP induces VEGF resistance in endothelial cells by increasing GPX1 and subsequently suppressing the VEGF-induced ROS generation that is required for VEGF signal transduction. This speculation was supported by our findings that the co-administration of BSO, an inhibitor of glutathione synthesis, rescued the inhibitory effects of SeP on VEGF signaling and the subsequent VEGF responsiveness. The identification of SeP receptor(s) in endothelial cells would provide further insight into the molecular mechanism by which SeP impairs VEGF signal transduction.

VEGF signaling is known to play paradoxical roles in the pathogenesis of diabetic complications. Both enhancement and suppression of angiogenesis are observed in different tissues in diabetic conditions [35]. In contrast to hindlimb ischaemia or wound healing, advanced diabetic retinopathy is characterized by VEGF-induced abnormal neovascularization in the retina. Current management for diabetic retinopathy includes anti-VEGF therapy along with blood glucose control [36]. In addition to retinopathy, growing evidence indicates that VEGF-related abnormal angiogenesis plays a major role in diabetic nephropathy [37]. Moreover, a recent report showed that pharmacological inhibition of VEGF-B improves glucose tolerance and insulin resistance in rodent models with type 2 diabetes [38]. Additional studies are needed to determine the actions of SeP on the enhanced angiogenesis in diabetic retinopathy or nephropathy.

Different from phosphorylation of VEGFR2 and ERK1/2, VEGF-induced phosphorylation of Akt, p38 MAPK, and AMPK was unchanged by SeP in HUVECs (data not shown). Although the detailed molecular mechanism by which SeP selectively impairs VEGFR2/ERK pathway in HUVECs is still unknown, SeP might act on ERK-selective MAPK phosphatases [39]. In fact, some kinds of MAPK phosphatases are inactivated by intracellular oxidative stress [39]. However, SeP-induced selective impairment of VEGFR2/ERK pathway should be confirmed in the other vascular endothelial cells except HUVECs.

All the culture media we used for HUVECs in this study contain 5.5 mM of glucose, which corresponds to fasting plasma glucose levels in people with normal glucose tolerance. However, we confirmed that SeP also attenuated VEGF signaling of HUVECs in the presence of 25 mM of glucose (data not shown). These results suggest that SeP induces VEGF resistance in HUVECs on both normoglycemic and hyperglycemic conditions. However, additional experiments are clearly needed to determine whether SeP sufficiently remove hyperglycemia-induced chronic oxidative stress in vascular endothelial cells.

We have shown that serum levels of total selenium were unchanged in the mice injected with *SEPP1* plasmid compared with the control animals (Fig. 4c), in spite of the significant elevation of serum SeP (Fig. 4b). Selenium content in the other forms except SeP might decrease in the serum of the SeP transgenic mice compensatively [14, 40]. Because a recent report showed that SeP exerts anti-oxidative actions independently of selenium supply [41], we speculate that the phenotype of the

SeP transgenic mice reflects the action of SeP itself, but not the abnormal selenium distribution in mice.

Sepp1-heterozygous knockout mice exhibit an increase of angiogenesis during hindlimb ischaemia without the induction of diabetes (Figure 5), suggesting that the hepatokine SeP plays a role in the regulation of systemic angiogenesis, irrespective of diabetes status. For example, lipopolysacchadire-induced acute inflammation was reported to down-regulate the production of SeP in mice [42]. Angiogenesis promoted by suppressed production of SeP might be beneficial in inflammatory conditions. Further characterization of *Sepp1*-deficient mice will provide insights into the involvement of SeP in the regulation of angiogenesis in normoglycemic condition.

Serum levels of human SeP in the mice injected with human *SEPP1* plasmid reached approximately 2.0 μg/ml (Fig. 4b). These levels correspond to the incremental changes of serum levels of SeP in people with normal glucose tolerance to those with type 2 diabetes in Japanese population [15, 25]. This strongly suggests that the phenotype observed in the SeP transgenic mice reflects the physiological actions of SeP.

One limitation of the present study is that we examined the action of SeP on endothelial cells only. Various types of cells participate in the angiogenic processes. Further studies are necessary to determine whether SeP exerts effects on other cell types such as the monocytes or endothelial progenitor cells.

Another limitation of the present study is that we carried out all the experiments of *Sepp1*-deficient

mice without the induction of diabetes by use of high fat diet or streptozotocin. Hence, we did not investigate the contribution of SeP in the development of the dysregulated angiogenesis seen in diabetes *in vivo*. However, our data indicate that treatment with purified SeP directly inhibits angiogenesis in both vascular endothelial cells and mice on euglycemic conditions. Combined with the previous reports showing the elevated production of SeP in type 2 diabetes [15, 16], the current data suggest that overproduction of SeP contributes to the onset of impaired angiogenesis in type 2 diabetes. However, further studies in animals with diabetes are necessary to determine the degree of the contribution of SeP on the impaired angiogenesis observed in diabetes.

In summary, the present study indicates that the diabetes-associated hepatokine SeP impairs angiogenesis by reducing VEGF signal transduction in endothelial cells, and suggests that SeP may be a novel therapeutic target for treatment of VEGF resistance in people with type 2 diabetes.

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Contribution statement K.I. researched the data, and wrote the manuscript. H.M. conceived and designed the experiments, researched the data, contributed to the discussion, wrote the manuscript, and reviewed and edited the manuscript. M.K. researched the data, contributed to the discussion, and reviewed and edited the manuscript. H.T., N.M-N., N.Tajima, K.C., F.L., H.A., T.O., M.S., Y.T., K.K., A.F. and K.M. designed the experiments, contributed to the discussion, and reviewed the manuscript. Y.S., Y.O., Y.T., K.T., H.K., S.Kameo and N.Takakura conceived and designed the experiments, researched the data, contributed to the discussion, and revised the manuscript critically for important intellectual content. S.Kaneko, and T.T. conceived and designed the experiments, contributed to the discussion, wrote the manuscript, and reviewed and edited manuscript. T.T. is the guarantor of this work, has full access to all the data in the study, and takes responsibility for the integrity of the data and accuracy of the data analysis. All the authors have approved the final version of the manuscript.

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Legends for figures

Fig. 1 Glucose increases gene expression and transcriptional activity for SeP in H4-II-EC3 hepatocytes. (**a**) Relative *Sepp1* mRNA expression normalized to b-Act, (**b**) Promoter activity for *SEPP1* in H4-II-EC3 hepatocytes treated with glucose (white) and mannitol (black). Data are mean ±SD, $n = 3$, ***$p < 0.001$

Fig. 2 SeP suppresses VEGF-stimulated angiogenesis in vascular endothelial cells. (**a**) Cell proliferation in HUVECs treated with VEGF for 48 hours ($n = 12$). (**b**) Representative images of HUVECs that migrated across the polycarbonate filters (magnification, x200). (**c**) Quantification of HUVECs that migrated across the filters ($n = 8$). (**d**) Representative images of HUVECs that were subjected to Matrigel tubule formation assay (magnification, x400). (**e**) Quantification of total tubule length of HUVECs ($n = 9$). (**f**) Matrigel implant assay in mice ($n = 6$-8). Black bar: Control; dark-grey bar: SeP 5 μg/ml; white bar: SeP 10 μg/ml; light-grey bar: SeP 10 μg/ml and BSO 0.2 mmol/l in (a), (c), (e) and (f). Data are mean ± SEM in (**a**), (**c**), (**d**), and (**f**). *$p < 0.05$. **$p < 0.01$.

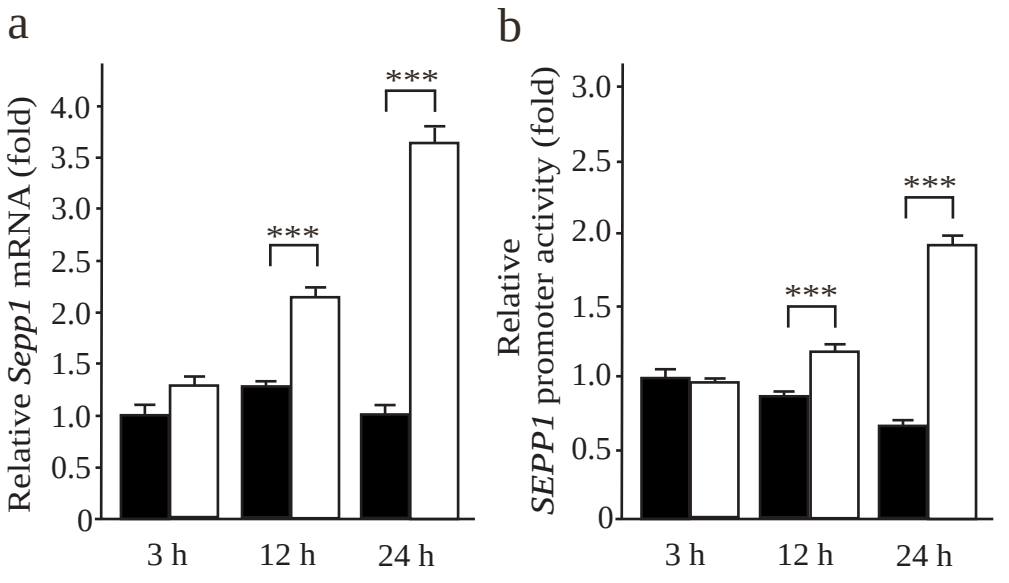
Fig. 3 SeP impairs VEGF signal transduction in endothelial cells. (**a**) VEGF signaling in HUVECs treated with SeP (10 μg/ml). (**b**) Quantification of phosphorylated VEGFR2 and ERK in HUVECs ($n = 6$). (**c**) Gene expression levels for VEGFR2 (KDR) in HUVECs treated with SeP for 24 hours normalized to GAPDH ($n = 6$). (**d**) ROS levels in HUVECs that were stimulated with VEGF for 5 min. ($n = 8$). ROS levels were measured as DCF fluorescence intensity. Black bar: Control; dark-grey bar: SeP 5 μg/ml; white bar: SeP 10 μg/ml; light-grey bar: SeP 10 μg/ml and BSO 0.2 mmol/l in (b)-(d). Data are mean ± SEM in (**b**)-(**d**). *$p < 0.05$, **$p < 0.01$.

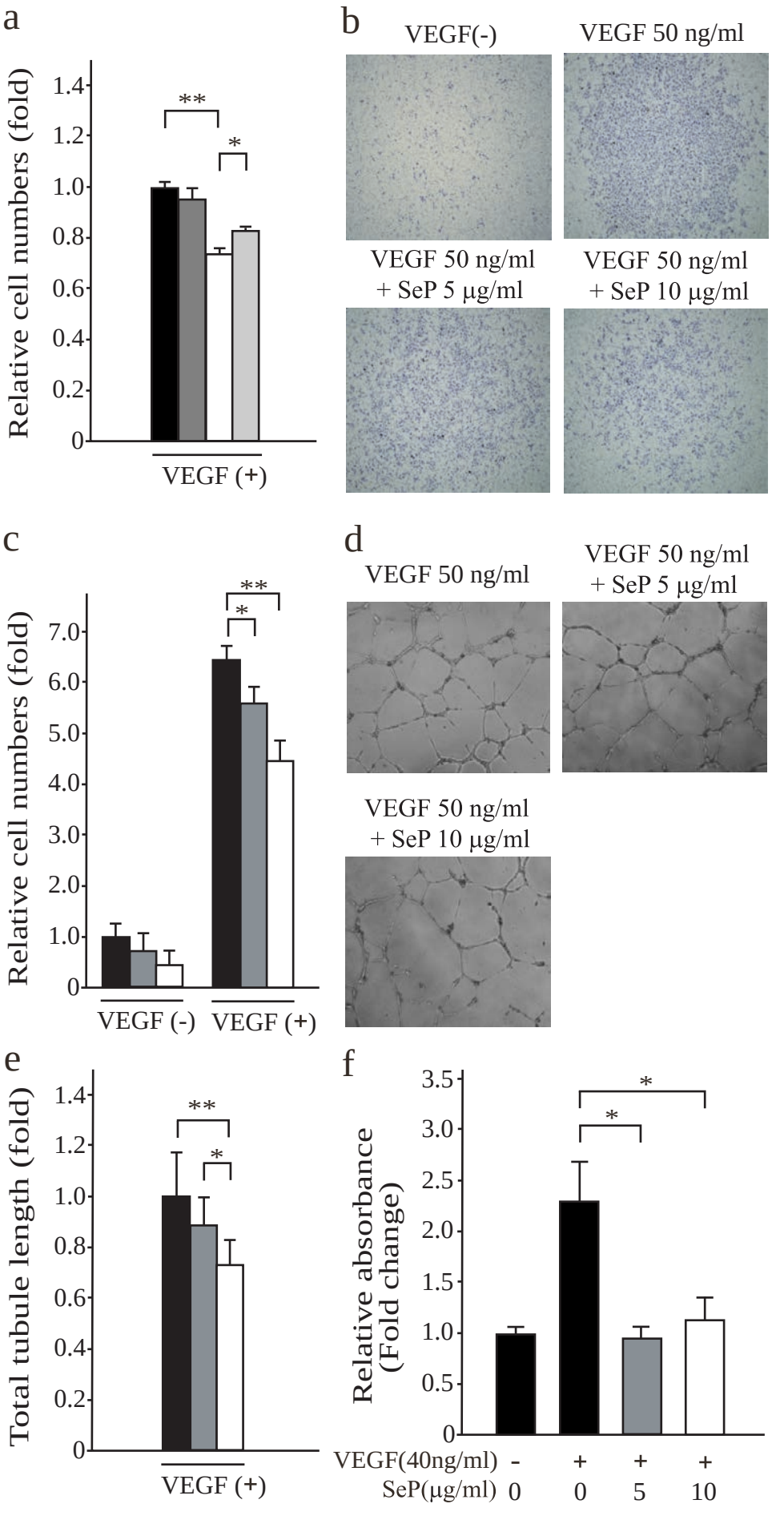
Fig. 4 Hepatic overexpression of SeP impairs wound healing in mice. (**a**) Levels of human *SEPP1* mRNA normalized to 18s rRNA in the livers of mice injected with plasmid DNA *via* the tail vein ($n = 9$). (b) Serum human SeP levels in mice injected with a plasmid encoding *SEPP1* ($n = 9$). (**c**) Serum levels of selenium in mice injected with a plasmid encoding *SEPP1* ($n = 3$). (**d**) Representative images of full-thickness excision rounds on the backs of mice injected with *SEPP1* plasmid. (**e**) Quantification of wound closure in mice injected with *SEPP1* plasmid (white circle) and control (black circle) ($n = 9$). (**f**) Representative images of full-thickness excisional wounds on the backs of *Sepp1*$^{-/-}$ mice. (**g**) Quantification of wound closure in *Sepp1*$^{-/-}$ mice (white circle) and control (black circle) ($n = 6$-12). Data are mean ± SEM in (**a**), (**b**), (c), (**e**), and (**g**). *$p < 0.05$, Scale bars: 10 mm in (**d**) and (**f**).

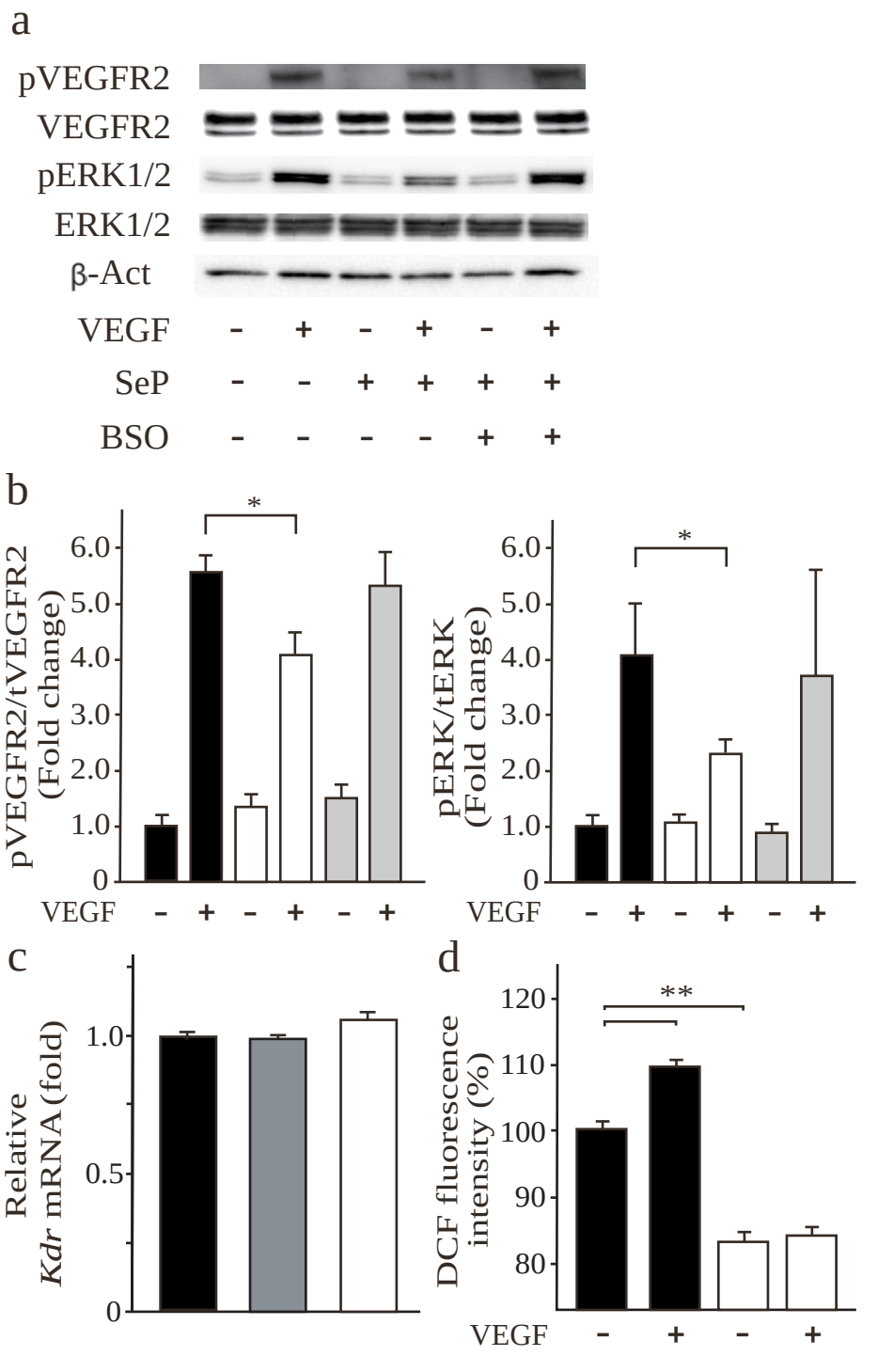
Fig. 5 *Sepp1*$^{+/-}$ mice show enhanced angiogenesis during hindlimb ischaemia. (**a**) Representative images of perfusion recovery following hindlimb ischaemia in *Sepp1*$^{+/-}$ mice. (**b**) Quantification of blood flow recovery in *Sepp1*$^{+/-}$ mice (white circle) and control (black circle) ($n = 5$). The ratio of

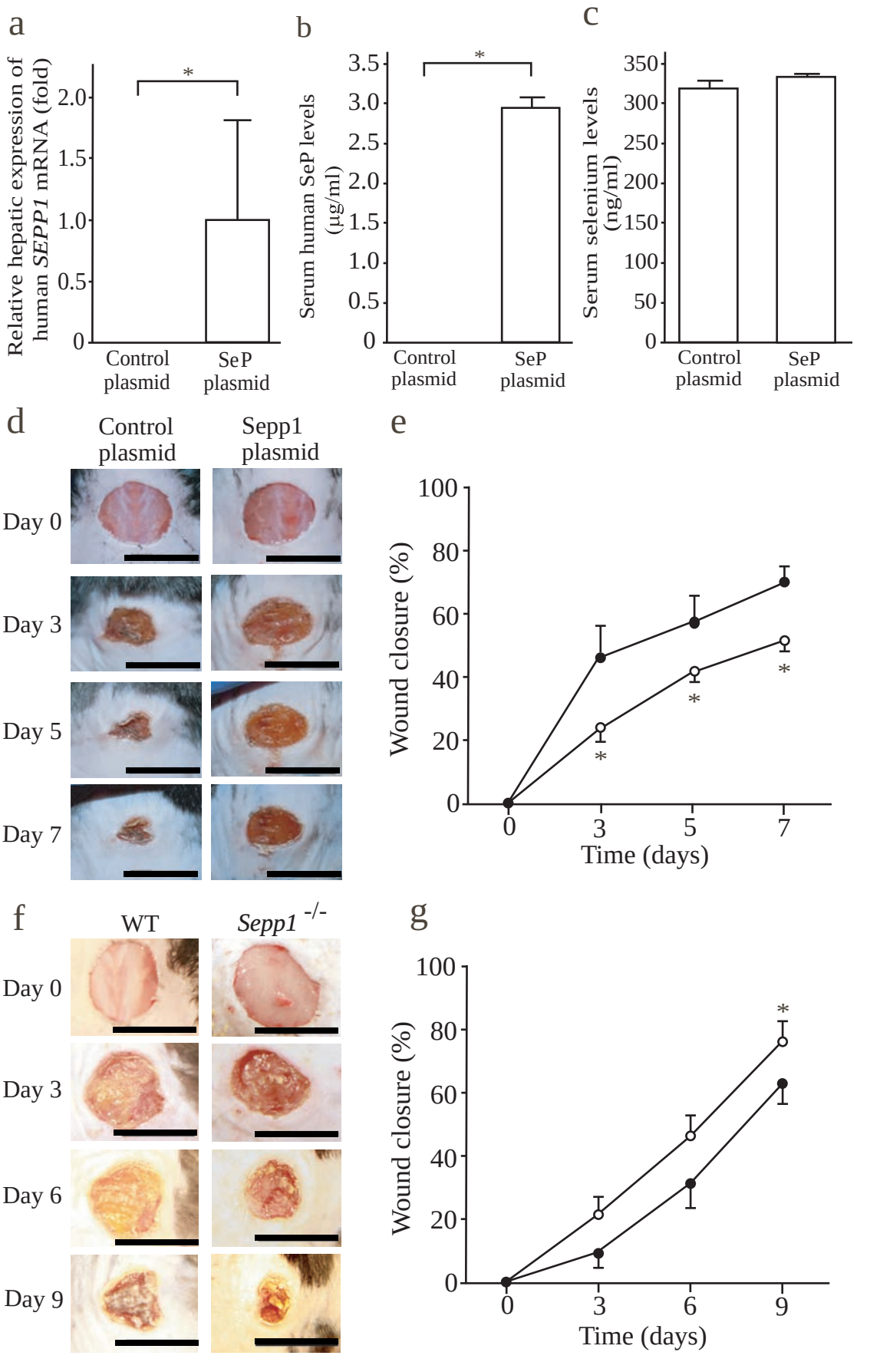
relative blood flow was calculated by dividing the laser Doppler-derived perfusion of the ischaemic hindlimb by non-ischaemic. (**c**) Representative images of CD31 stained sections of lower limb tissues of *Sepp1*$^{+/-}$ mice in 15 days after ligation. Scale bars: 100 μm (**d**) Quantification of CD31-positive cells in the hindlimb of *Sepp1*$^{+/-}$ mice (white bar) and WT (black bar). Data are from 16 fields per section. Data are mean ± SEM in (**b**), and (**d**). *$p < 0.05$, **$p < 0.01$.

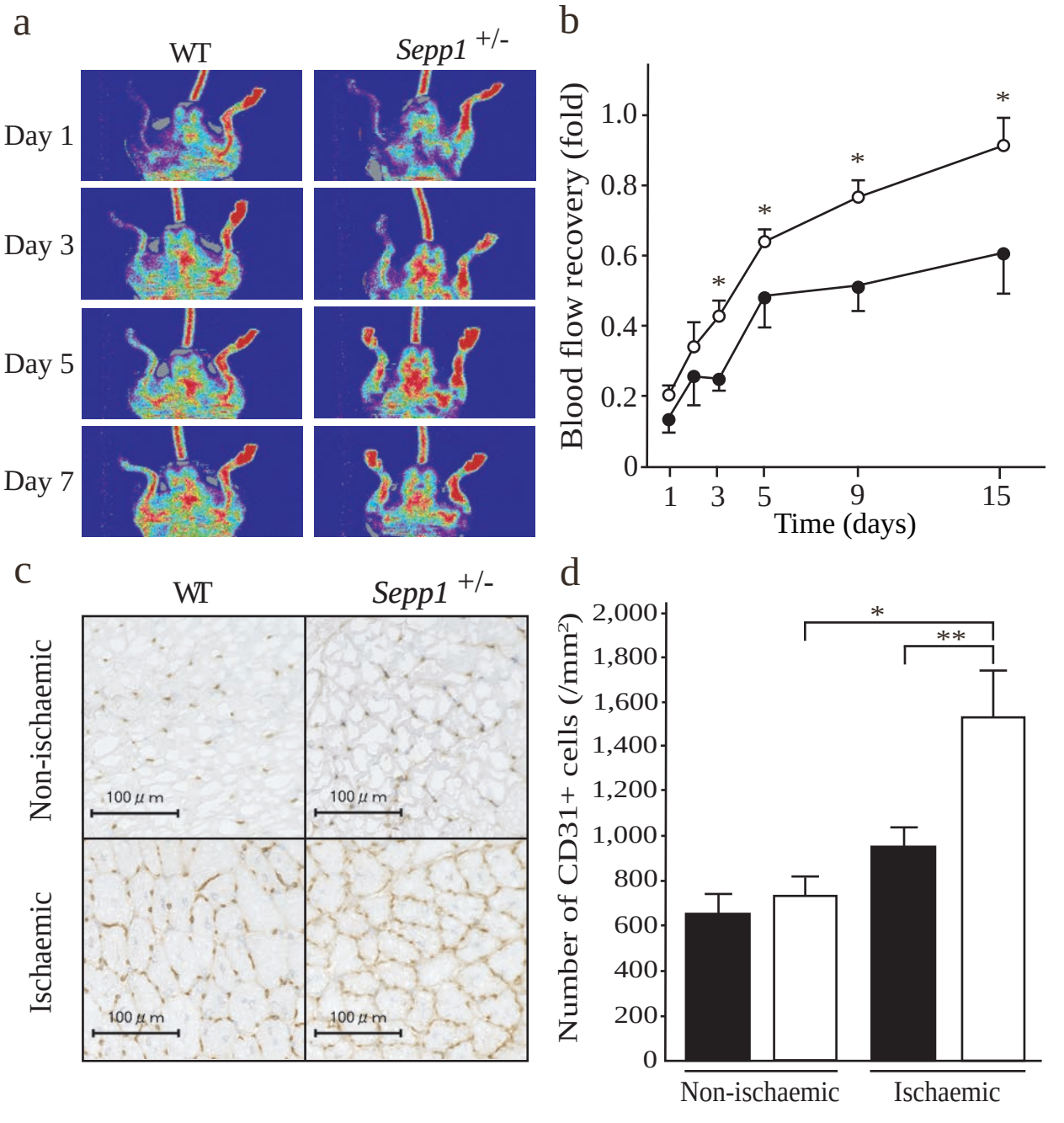
Fig. 6 Overproduction of SeP in Type 2 diabetic liver induces VEGF resistance in vascular endothelial cells. (**a**) SeP inhibits VEGF signal transduction by suppressing acute generation of ROS, resulting in the onset of impaired angiogenesis.


a
Relative Sepp1 mRNA (fold)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
***
***
3 h
12 h
24 h
b
Relative
SEPP1 promoter activity (fold)
3.0
2.5
2.0
1.5
1.0
0.5
0
***
***
3 h
12 h
24 h

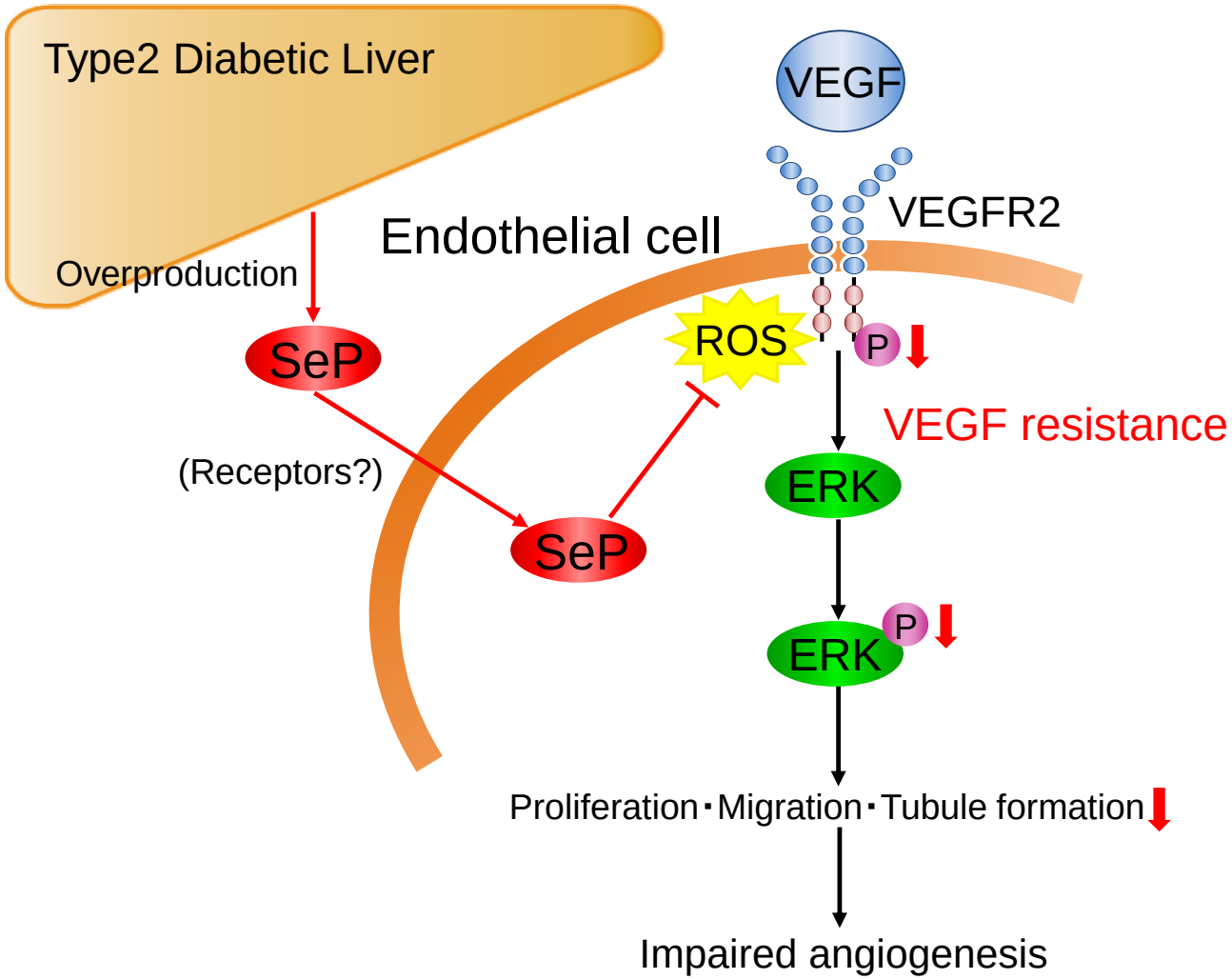

a
Relative cell numbers (fold)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
**
*
VEGF (+)
b
VEGF(-)
VEGF 50 ng/ml
VEGF 50 ng/ml
+ SeP 5 μg/ml
VEGF 50 ng/ml
+ SeP 10 μg/ml
c
Relative cell numbers (fold)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
**
*
VEGF (-)
VEGF (+)
d
VEGF 50 ng/ml
VEGF 50 ng/ml
+ SeP 5 μg/ml
VEGF 50 ng/ml
+ SeP 10 μg/ml
e
Total tubule length (fold)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
**
*
VEGF (+)
f
Relative absorbance
(Fold change)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
*
*
VEGF(40ng/ml) - + + +
SeP(μg/ml) 0 0 5 10


a
pVEGFR2
VEGFR2
pERK1/2
ERK1/2
β-Act
VEGF - + - + - +
SeP - - + + + +
BSO - - - - + +
b
pVEGFR2/tVEGFR2 (Fold change)
6.0
5.0
4.0
3.0
2.0
1.0
0
*
VEGF - + - + - +
pERK/tERK (Fold change)
6.0
5.0
4.0
3.0
2.0
1.0
0
*
VEGF - + - + - +
c
Relative Kdr mRNA (fold)
1.0
0.5
0
d
DCF fluorescence intensity (%)
120
110
100
90
80
**
VEGF - + - +


a
Relative hepatic expression of human SEPP1 mRNA (fold)
2.0
1.5
1.0
0.5
0
*
Control plasmid
SeP plasmid
b
Serum human SeP levels (μg/ml)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
*
Control plasmid
SeP plasmid
c
Serum selenium levels (ng/ml)
350
300
250
200
150
100
50
0
Control plasmid
SeP plasmid
d
Control plasmid
Sepp1 plasmid
Day 0
Day 3
Day 5
Day 7
e
Wound closure (%)
100
80
60
40
20
0
0
3
5
7
Time (days)
*
*
*
f
WT
Sepp1 -/-
Day 0
Day 3
Day 6
Day 9
g
Wound closure (%)
100
80
60
40
20
0
0
3
6
9
Time (days)
*


a
WT
Sepp1 +/-
Day 1
Day 3
Day 5
Day 7
b
Blood flow recovery (fold)
1.0
0.8
0.6
0.4
0.2
0
1
3
5
9
15
Time (days)
*
c
WT
Sepp1 +/-
Non-ischaemic
Ischaemic
100 μm
d
Number of CD31+ cells (/mm2)
2,000
1,800
1,600
1,400
1,200
1,000
800
600
400
200
0
*
**
Non-ischaemic
Ischaemic

a


Type2 Diabetic Liver
Overproduction
SeP
(Receptors?)
SeP
Endothelial cell
VEGF
VEGFR2
ROS
P
VEGF resistance
ERK
P
ERK
Proliferation・Migration・Tubule formation
Impaired angiogenesis

ESM Methods

Materials The materials used and their sources were as follows: VEGF (Pepro Tech, London, UK), HuMedia EG2 (Kurabo, Osaka, Japan), EBM-2 (Takara Bio, Otsu, Japan) and M199 medium (Gibco, Carlsbad, CA, USA), DMEM (Gibco), Matrigel (Chemicon, Billerica, MA, USA), BSO, Bovine serum albumin (BSA) and fatty acid free BSA (Sigma-Aldrich, St Louis, MO, USA), FBS (JRH Biosciences, St Louis, MO, USA), antibodies against phospho-VEGFR2 (Tyr1175), VEGFR2, phospho-ERK1/2 (Thr202/Tyr204), ERK1/2, phospho-Akt (Ser473), Akt, phospho-p38MAPK, p38MAPK antibody (Cell Signaling Technology, Boston, MA, USA), and CD31 antibody (BD, San Jose, CA, USA). Unless otherwise indicated, all other chemicals were purchased from Sigma-Aldrich.

Animals Male C57BL/6J mice, 6weeks of age, were purchased from Charles River Laboratories Japan (Yokohama, Japan). *Sepp1*-deletion mice were produced by homologous recombination with genomic DNA cloned from a Sv-129 P1 library, as described previously [1]. All animal studies were reviewed and approved by the committee for animal study at our institute, and were conducted in accordance with established standards for human handling.

Measurement of selenium Total selenium concentrations in mouse serum and culture medium were determined by modification of Watkinson's method [2,3]. The method was based on the measurement of the fluorescence of piazselenol resulted from reaction of selenite with 2,3-diaminonaphtalene

(DAN). The pretreated samples were measured fluorometrically (emission at 525 nm upon excitation at 378 nm) with a HITACH F2700 fluorescence spectrophotometer (HITACHI High-Technologies, Tokyo, Japan). The accuracy of the selenium analysis was checked by measurement of the reference material, Seronorm Trace Elements Serum L-2, REF 203105, LOT 0903107 (SERO, Billingstad, Norway). The average of obtained results (163.75 μg/ml) of the reference material serum fell within the recommended range (153-173 μg/ml) by SERO.

SEPP1 promoter assay The human *SEPP1* promoter region was cloned to a luciferase reporter vector as were ported previously [4]. H4-II-EC3 hepatocytes were grown to 40-50% in 24-well plates and transfected with 0.4 μg of plasmid DNA encoding a luciferase reporter vector per well together with 1.2-μl FuGENE6 (Promega, Madiosn, WI, USA). 24 hours later, the cells were washed with phosphate-buffered saline and starved in glucose free DMEM (Gibco) for 3 hours. Then, the cells were treated with 25 mmol/l of glucose or mannitol diluted with glucose free DMEM for the indicated times. Luciferase activities were measured using the Dual Luciferase assay system (Promega, Madison, WI, USA), as we described previously [4].

Cell proliferation assay HUVECs were seeded on 96-well plates (BD) at a density of 2×10^3 cells/well in 100 μl. After 3 hours of starvation in growth factor-free HuMedia at 37°C, HUVECs were incubated in HuMedia with VEGF (20 ng/ml) and SeP for 48 hours. Cell numbers were quantified by measuring

optical density at 450 nm using Cell Counting Kit-8 (Wako, Osaka, Japan).

Migration assay Cell Migration was assessed using polycarbonate filters with a pore size of 8 μm (Neuro Probe, Inc., Gaithersburg, MD, USA). HUVECs were pretreated with SeP in M199 containing 1% FBS for 24 hours. The HUVECs were then starved in EBM-2 containing 0.5% BSA for 3 hours. The indicated concentrations of VEGF and SeP in EBM-2 containing 0.25% fatty acid free BSA were placed in the lower chamber of the filter. HUVECs (1×10^4) in 36 μl of EBM-2 containing 0.25% fatty acid-free BSA were seeded in the upper chamber. The apparatus was incubated for 15 hours. The migrated cell number across the filter was counted in 16 high-power (×200) fields per insert.

Cell tubule formation assay 96-well plates were coated with ECMatrix gel, and the Matrigel was allowed to solidify for 60 min at 37°C. HUVECs (1×10^4) in 100 μl of M199 medium containing 5% FBS with various concentrations of SeP were seeded onto each well. After incubation at 37°C for 11 hours, endothelial tubule formation was photographed under microscope. Tubule length was measured with Image J software to quantify the length of tubular structures.

Matrigel plug implantation assay 20 μl plug of Matrigel containing VEGF (40 ng/ml) with SeP were planted into the subcutaneous tissue on the back of WT mice. On day 10 after implantation, the Matrigel plug was removed and gel was collected by centrifugation. The collected cells were labeled

with FITC-lectin. Angiogenesis was quantified by measuring fluorescence intensity in 96-well plates (excitation, 485 nm; emission, 510 nm).

Western blot analysis HUVECs were pretreated with SeP for 24 hours. After 2 hours of starvation with serum-free M199 medium, HUVECs were stimulated with VEGF (20 ng/ml) for 15 min. Cells were lysed immediately with modified RIPA buffer. Lysates (containing 6-15 μg of protein) were immunoblotted with antibodies to phospho-VEGFR2 (Tyr1175), phospho-ERK1/2 (Thr202/Tyr204), ERK1/2, or actin. Immunoreactive bands were detected using ECL Prime and were visualized using LAS-3000 (FUJIFILM, Tokyo, Japan).

RNA preparation and quantitative real-time PCR Total RNA was isolated from frozen liver with a QuickGene RNA Tissue Kit SII (FUJIFILM). Total RNA (100 ng) was used to synthesize cDNA with a High-Capacity cDNA Archive kit (Applied Biosystems, Carlsbad, CA, USA). Real-time PCR was performed on an ABI-Prism 7900HT (Applied Biosystems). The specific PCR primers and TaqMan probe were obtained from Applied Biosystems. The PCR conditions were one cycle of 50℃ for 2 min and 95℃ for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min.

ROS generation Intracellular HUVECs were pretreated with SeP for 24 hours in HuMedia EG2. After 2 hours of starvation with serum-free M199 medium and 30 min of treatment with DCF, HUVECs

were stimulated with VEGF (50 ng/ml) for 5 min. ROS generation was quantified by Fluorescent plate reader (Fluoroskan Ascent FL, Yokohama, Japan).

Purification of SeP SeP was purified from human plasma using conventional chromatographic methods, as previously described [5,6]. Homogeneity of purified human SeP was confirmed by analysis of both amino acid composition and sequence [6]. Concentrations of purified SeP were measured by the Bradford method, using bovine immunoglobulin G as a standard.

Preparation of human SEPP1 plasmid and overexpression of SeP in mice The human *SEPP1* gene was cloned into the pBR322 expression vector, and control plasmid DNA was also produced. 55 μg of human *SEPP1* pDNA or control pDNA in 1.8 ml of phosphate-buffered saline (pH 7.4, $CaCl_2$-, $MgCl_2$-) were injected within 10 s into the tail vein of male C57BL/6J mice weighing 25 g each using a 26G needle. Two days after injection, *SEPP1* mRNA expression in the liver of mice was quantified by real-time PCR.

Mouse wound healing model A circle of 10 mm in diameter was drawn on the skin of the mid-dorsal region, and a full-thickness wound was created by excision of the area with scissors. The extent of wound closure was examined after wounding.

Hindlimb ischaemia model Mice underwent ligation and segmental resection of the left femoral vessel as described previously [7]. The blood flow was measured with a laser-Doppler perfusion scanner. The ratio of the relative blood flow was calculated by dividing the perfusion of the ischaemic hindlimb by that of the non-ischaemic.

Identification of $CD31^+$ vessels 5-μm sections of muscle were acetonized and incubated with primary antibody to CD31 (PECAM-1, BD557355; 1:100) (BD) overnight at 4°C. The sections were then incubated with a rat-specific antibody. Immunohistochemical signals were developed with 3, 3'-diaminobenzidine substrate.

Calculations and statistical analysis Numeric values are reported as mean ± SEM. Differences between two groups were assessed with the unpaired two-tailed *t*-tests. Data involving more than two groups were assessed by analysis of variance (ANOVA) with Bonferroni adjustment for multiple comparisons. In all analyses, $P<0.05$ was taken to indicate statistical significance.

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