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Activation of PDGF-CC by tissue plasminogen activator impairs blood-brain barrier integrity during ischemic stroke

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Thrombolytic treatment of ischemic stroke with tissue plasminogen activator (tPA) is markedly limited owing to concerns about hemorrhagic complications and the requirement that tPA be administered within 3 h of symptoms. Here we report that tPA activation of latent platelet-derived growth factor-CC (PDGF-CC) may explain these limitations. Intraventricular injection of tPA or active PDGF-CC, in the absence of ischemia, leads to significant increases in cerebrovascular permeability. In contrast, co-injection of neutralizing antibodies to PDGF-CC with tPA blocks this increased permeability, indicating that PDGF-CC is a downstream substrate of tPA within the neurovascular unit. These effects are mediated through activation of PDGF-α receptors (PDGFR-α) on perivascular astrocytes, and treatment of mice with the PDGFR-α antagonist imatinib after ischemic stroke reduces both cerebrovascular permeability and hemorrhagic complications associated with late administration of thrombolytic tPA. These data demonstrate that PDGF signaling regulates blood-brain barrier permeability and suggest potential new strategies for stroke treatment.

Stroke is a leading cause of adult morbidity and mortality[1]. Ischemic stroke is the most common form of stroke and occurs when there is an abrupt interruption of blood flow to the brain[2]. The only drug currently approved by the US Food and Drug Administration specifically for ischemic stroke is the thrombolytic agent tPA. However, there is only a narrow window during which thrombolysis can be safely accomplished, and treatment of patients with tPA more than 3 h after the onset of symptoms is not recommended. Thus, fewer than 3% of potential patients currently receive tPA[1,3]. The limited benefit of tPA may be due to its previously unidentified activities in the central nervous system (CNS) beyond its well established thrombolytic role.

tPA is a highly specific serine protease that cleaves and thereby activates the zymogen plasminogen into the broad-specificity protease plasmin. Outside of the CNS, tPA is primarily a thrombolytic enzyme, as plasmin's principal substrate is fibrin. However, within the CNS, the role of tPA is not well characterized, and its primary substrates are not known. A growing body of evidence indicates that tPA is involved in pathological events in the CNS including neurodegeneration, seizures and cerebral ischemia[4–12]. The role of endogenous tPA in cerebral ischemia is complex, with the potential for both beneficial and harmful effects[6,13]. Early experiments in animal models of stroke showed that thrombolytic tPA reduced neurological damage when given within a few hours of the onset of ischemia[14]. Likewise, in humans, early tPA treatment of ischemic stroke results in a 30% increase in the number of subjects with minimal disability at three months[15]. In contrast, recent studies in animal models of transient and permanent middle cerebral artery occlusion (MCAO) showed that both genetic deficiency[6,7] and inhibition[8,10,16] of tPA within the CNS are associated with improved stroke outcome. The mechanisms of tPA's harmful effects in stroke are unknown, but it has been suggested that tPA within the CNS may intensify ischemia-induced excitotoxicity[6]. tPA can also directly affect the integrity of the blood-brain barrier (BBB)[17–19], and, in one study, the late administration of thrombolytic tPA significantly increased BBB dysfunction 24 h after embolic stroke[16]. This suggests that some of tPA's negative effects after stroke may be secondary to its role in ischemia-induced BBB dysfunction.

Previously, we showed that tPA is both necessary and sufficient to induce opening of the BBB[17]. This effect required proteolytically active tPA but was independent of plasminogen, implicating another substrate for tPA. Recently, we identified the dimeric growth factor

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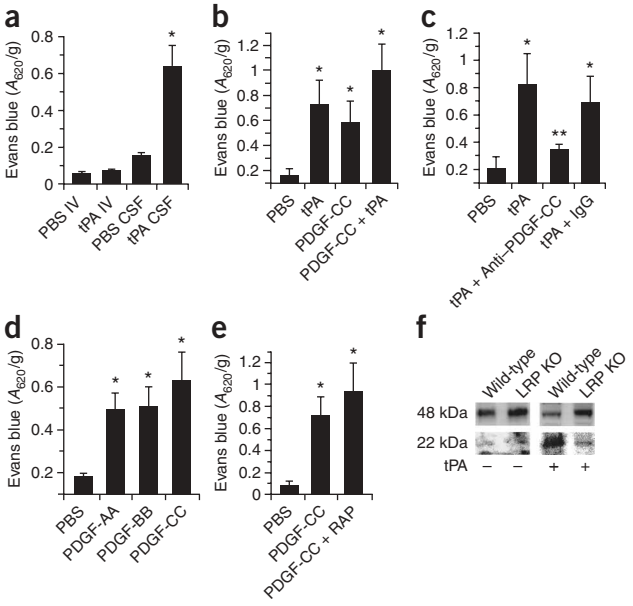

Figure 1 Active PDGF-CC mediates tPA-induced cerebrovascular permeability. (**a**) Comparison of Evans blue extravasation 1 h after either intravenous injection (IV) with 10 mg/kg of tPA as a bolus (~250 μg/mouse) or intraventricular injection (CSF) with 585 ng of tPA. (**b**) Evans blue extravasation 1 h after intraventricular injection of PBS, tPA, active PDGF-CC or active PDGF-CC together with tPA (PDGF-CC + tPA). (**c**) Evans blue extravasation 1 h after intraventricular injection of either PBS, active tPA, tPA with blocking antibodies to PDGF-CC (tPA + anti–PDGF-CC) or tPA together with control IgG (tPA + IgG). (**d**) Evans blue extravasation 1 h after intraventricular injection of PBS, PDGF-AA, PDGF-BB, or active PDGF-CC. (**e**) Evans blue extravasation 1 h after intraventricular injection with either PBS, active PDGF-CC or active PDGF-CC together with the LRP antagonist RAP (PDGF-CC + RAP). For all injections into the CSF, 3 μl of 3 μM protein was used except for antibodies, which were 0.4 mg/ml. For each group, $n = 8$–10 and data represent means ± s.e.m. $^*P < 0.01$ versus PBS and $^{**}P < 0.05$ versus the IgG control. (**f**) PDGF-CC cleavage by tPA is impaired in *Lrp1*$^{-/-}$ cells (LRP KO). Serum-free medium from *Lrp1*$^{-/-}$ MEFs and wild-type cells shows that both cell lines express the 48-kDa full-length PDGF-C, whereas the addition of exogenous tPA to the cells only generates the 22-kDa PDGF-C species in the presence of the wild-type cells but not in *Lrp1*$^{-/-}$ cells.

PDGF-CC as a new substrate for tPA[20]. The monomeric PDGF-C protein has a two-domain structure, and tPA cleavage of the N-terminal CUB domains from latent PDGF-CC generates active PDGF-CC capable of triggering PDGFR-α signaling[21]. Therefore, we hypothesized that PDGF-CC is a candidate substrate for tPA that may regulate BBB integrity in the CNS.

RESULTS

tPA increases cerebrovascular permeability through PDGF-CC

The demonstration that tPA injection into the cerebrospinal fluid (CSF) rapidly increases cerebrovascular permeability[17] led us to propose that tPA regulates cerebrovascular permeability through a substrate-dependent, receptor-mediated cell signaling event within the neurovascular unit. The neurovascular unit is composed of endothelial cells, astrocytes, microglia, neurons and smooth muscle cells or pericytes. To determine whether tPA acts specifically on the CNS side of the neurovascular unit, we compared the effects of tPA injected into the CSF with the effects of intravenously administered tPA on Evans blue dye extravasation in nonischemic mice. This analysis revealed that tPA acts on the abluminal side of the neurovascular unit, as increased cerebrovascular permeability was observed only when enzymatically active tPA was injected into the CSF and not when it was administered intravenously (**Fig. 1a** and **Supplementary Fig. 1** online). Because tPA activates latent PDGF-CC[20,22], we examined whether active PDGF-CC also induced cerebrovascular permeability. Like tPA, the intraventricular injection of active PDGF-CC significantly ($P < 0.01$) increased cerebrovascular permeability to an extent that was similar to that observed with tPA (**Fig. 1b**). The combined treatment with both tPA and PDGF-CC was not significantly different from either treatment alone, indicating no additive or synergistic increase in cerebrovascular permeability with the two agents. This suggests that tPA and PDGF-CC may act within a common pathway.

To test whether PDGF-CC was acting downstream of tPA, we co-injected neutralizing antibodies against PDGF-CC (**Supplementary Fig. 2** online) with tPA. These data show that PDGF-CC–specific antibodies, but not preimmune IgG, significantly ($P < 0.05$) inhibited the tPA-induced increase in cerebrovascular permeability (**Fig. 1c**). This indicates that PDGF-CC acts downstream of tPA and also suggests that tPA activates latent PDGF-CC within the CNS.

PDGF-CC is a known agonist of the PDGFR-α[21]. Therefore, we tested whether two other PDGFR-α agonists, PDGF-AA and PDGF-BB[23], also increased cerebrovascular permeability when injected into CSF. Both PDGF-AA and PDGF-BB increased Evans blue extravasation significantly ($P < 0.01$) compared to the PBS-treated group, demonstrating a PDGFR-α dependent process (**Fig. 1d**). However, in contrast to PDGF-CC–specific antibodies, neutralizing PDGF-AB–specific antibodies had no effect on tPA-mediated enhancement of BBB permeability (**Supplementary Fig. 3** online).

Several studies have shown that tPA-induced cerebrovascular permeability is inhibited by antagonists of members of the low-density lipoprotein receptor family (LDLRs)[17,19,24]. Therefore, we examined whether the LDLR antagonist, the receptor-associated protein (RAP) also inhibited PDGF-CC–induced cerebrovascular permeability. In contrast to its inhibition of tPA-induced Evans blue extravasation[17], RAP had no effect on the ability of active PDGF-CC to increase cerebrovascular permeability (**Fig. 1e**). This implies that active PDGF-CC does not require LDLRs to mediate its effect on the neurovascular unit, suggesting that, like tPA, the LDLRs act upstream of active PDGF-CC. Because both tPA and PDGF-CC bind the low-density lipoprotein receptor–related protein (LRP; **Supplementary Fig. 4** online), we speculated that LRP may facilitate activation of PDGF-CC by tPA. To test this, we examined tPA-mediated cleavage of PDGF-CC in the presence of mouse embryonic fibroblasts (MEFs) that either express LRP or are genetically deficient in LRP[25]. The results show that both cell types express similar amounts of latent PDGF-CC, but only cells expressing LRP stimulate efficient activation of PDGF-CC by tPA (**Fig. 1f**). This suggests that *in vivo* cell-associated LRP can markedly enhance PDGF-CC activation by tPA.

Analysis of vascular morphology by electron microscopy 1 h after intraventricular injection of either tPA or active PDGF-CC indicated that both proteins induced specific morphological changes in cerebral vessels compared to control injection (**Fig. 2**). In control tissue, vessels were intact and tightly associated with surrounding tissue (**Fig. 2a**,**d**). In contrast, arterioles from mice receiving intraventricular injections of tPA or PDGF-CC were surrounded by open areas of apparent fluid accumulation (**Fig. 2b**,**c**,**e**,**f**). This phenotype was observed in multiple arterioles in tPA- or PDGF-CC–treated mice but not in brain capillaries (**Fig. 2b**,**c**). The similarity in morphological changes

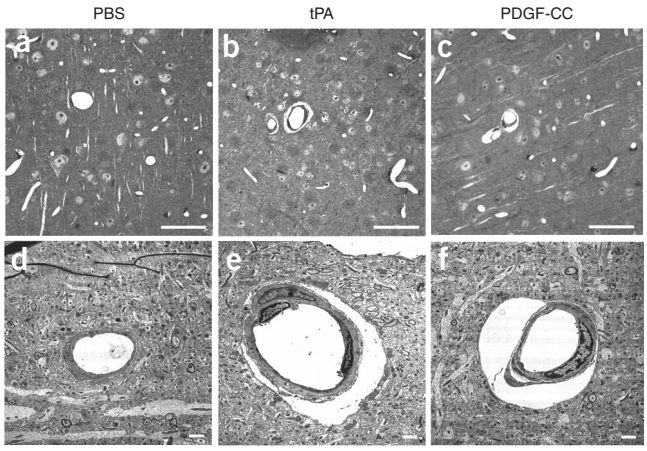

Figure 2 tPA and PDGF-CC induce similar morphological changes in brain vasculature. (**a**–**c**) Light microscopy images of cerebral sections prepared for electron microscopy analysis but stained with toluene blue. (**d**–**f**) High-magnification micrographs from electron microscopy of cerebral arterioles. Brains were harvested 1 h after intraventricular injection of either PBS (**a**,**d**), tPA (**b**,**e**) or PDGF-CC (**c**,**f**). Scale bars, 50 μm (**a**–**c**) and 2 μm (**d**–**f**).

observed in tPA- and PDGF-CC–treated mice along with the similar degree of increased cerebrovascular permeability induced by both agents suggests that PDGF-CC and tPA provoke comparable vascular changes that occur primarily in arterioles.

PDGF-CC, tPA and PDGFR-α in the neurovascular unit

Immunohistochemical staining showed that PDGF-CC localization throughout the brain closely resembles the reported expression of tPA[26], with the most abundant staining observed in cortex, striatum and hippocampus (data not shown). Higher resolution microscopy indicated that PDGF-CC was often closely associated with arterioles, but not with capillaries, and was typically seen as a stained patch on one side of larger vessels (**Fig. 3a**). Immunohistochemical analysis of tPA expression showed abundant vessel-associated tPA throughout the brain (data not shown). In capillaries, the staining was largely confined to endothelial cells, whereas in arterioles, tPA was also associated with perivascular cells (**Fig. 3b**). Thus, both PDGF-CC and tPA were located in the neurovascular unit of arterioles. We also investigated the expression of PDGF-CC and its receptor PDGFR-α in brains of mice doubly heterozygous for a nuclear-targeted histone H2B–GFP fusion protein inserted into the *Pdgfra* locus[27] (**Supplementary Fig. 5** online) and a lacZ reporter inserted into the *Pdgfc* locus[28]. Brains from the $Pdgfra^{+/GFP}Pdgfc^{+/lacZ}$ mice showed both PDGFR-α and PDGF-CC expression in cells associated with arterioles (**Fig. 3c**). PDGF-CC expression was also seen in cells not directly associated with vessels (**Fig. 3c**).

To further characterize PDGFR-α–positive vessels, we isolated GFP-positive vessel fragments from brain. The most intensely GFP-positive vessels resembled arterioles and were covered with firmly attached GFP-expressing cells (**Fig. 3d**,**e**). Whole-mount immunofluorescence staining of these vessel fragments for smooth muscle actin (SMA) revealed that the vessels were indeed arterioles, as they were strongly positive for SMA (**Fig. 3d**). However, the GFP-positive nuclei did not appear to directly colocalize with SMA and instead localized to the outside of the SMA-positive cells. In contrast, staining of the GFP-positive vessels for the astrocyte marker, glial fibrillar acidic protein (GFAP), showed that GFP-expressing nuclei appeared to be embedded in the GFAP-positive cells (**Fig. 3e**). To confirm these observations, we

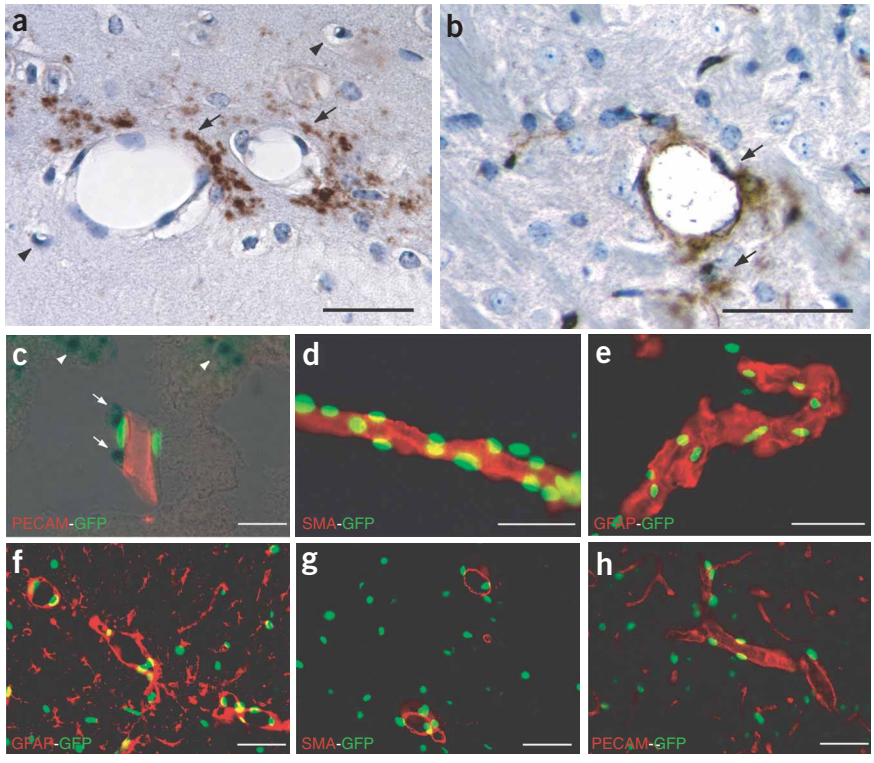

Figure 3 PDGF-CC, tPA and the PDGFR-α are expressed in the neurovascular unit. (**a**) Sections from normal mouse brains stained with antibodies to PDGF-CC show PDGF-CC staining in patches associated with arterioles (arrows) but not with capillaries (arrowheads). (**b**) Sections from normal mouse brains stained with antibodies to tPA show mainly perivascular tPA staining in association with an arteriole (arrows). (**c**–**h**) Micrographs showing PDGFR-α expression in mouse brain using a GFP reporter. (**c**) Sections of double heterozygous $Pdgfra^{+/GFP}$-$Pdgfc^{+/lacZ}$ mouse brains stained with the PDGF-C reporter X-gal and antibodies to the endothelial cell marker PECAM (PECAM, red). The arrows indicate vessel-associated PDGF-C expression, and arrowheads indicate non–vessel-associated expression. GFP and PECAM staining was visualized by fluorescence, whereas X-gal staining was viewed in bright field. (**d**,**e**) Whole-mount immunofluorescence staining of GFP-positive vessel fragments stained for SMA (red, **d**) confirmed that the GFP-positive vessel fragments are arterioles. The GFP-positive nuclei are mainly localized outside of the SMA-positive cells. In contrast, co-staining with GFAP (red; **e**), an astrocyte marker, suggests that the GFP-positive cells are astrocytes. (**f**–**h**) Immunofluorescence staining of brain sections from $Pdgfra^{+/GFP}$ mice stained with markers for astrocytes, (GFAP in red; **f**), vascular smooth muscle cells (SMA in red; **g**),and endothelial cells (PECAM in red; **h**). Similar to the isolated vessel fragments, colocalization of GFP expression with GFAP was abundant in the stained brain sections and produced a yellow color (**f**). Scale bars, 50 μm (**a**,**b**, **d**–**h**) and 20 μm (**c**).

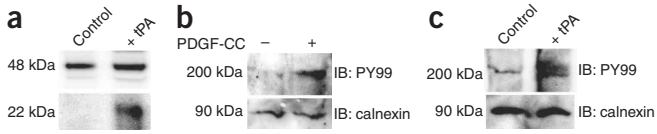

Figure 4 PDGF-CC is expressed by astrocytes in culture. (**a**–**c**) Immunoblot analysis of PDGF-CC expression in astrocytes. (**a**) A 48-kDa band corresponding to full-length PDGF-C is seen in control cell media, whereas addition of tPA to the cells before collection of the medium induces release of the active 22-kDa PDGF-C species. PDGFR-α expressed by astrocytes in culture is stimulated by addition of active PDGF-CC (**b**) or tPA (**c**) to the cells. Receptor tyrosine phosphorylation is determined by immunoblotting (IB) of cell lysates for phosphotyrosine. Immunoblotting for calnexin is also shown as a loading control.

stained intact brain sections from GFP-expressing mice by immunofluorescence for GFAP, SMA or platelet-endothelial cell adhesion molecule (PECAM, a marker of endothelial cells) and imaged together with GFP (**Fig. 3f–h**). These results showed that PDGFR-α largely colocalized with perivascular GFAP-positive cells (**Fig. 3f**) and not with cells expressing SMA (**Fig. 3g**) or PECAM (**Fig. 3h**), demonstrating that perivascular astrocytes are the primary neurovascular cells expressing PDGFR-α. These data suggest that tPA, PDGF-CC and PDGFR-α are localized to arterioles in the CNS, where they interact on the surface of perivascular astrocytes.

Because LRP is also known to be specifically associated with arteriolar perivascular astrocytes[29], we tested whether type I fibrous astrocytes in culture could enhance the activation of PDGF-CC by tPA and whether active PDGF-CC could stimulate PDGFR-α phosphorylation. These studies showed that astrocytes in culture expressed latent PDGF-CC that was cleaved by added tPA (**Fig. 4a**). Active PDGF-CC also stimulated PDGFR-α phosphorylation in astrocytes (**Fig. 4b**), as did the addition of tPA, suggesting a possible autocrine effect of PDGF-CC on astrocytes (**Fig. 4c**).

PDGFR-α regulates cerebrovascular permeability after stroke

To examine whether PDGFR-α was activated in a tPA-dependent manner during cerebral ischemia, we developed a mouse photothrombotic model of stroke that is similar to a previously described model[30,31]. This model permits thrombolytic treatment and can be analyzed either with or without reperfusion (**Supplementary Fig. 6** online). Six hours after unilateral MCAO in wild-type and tPA-deficient mice, we divided isolated brains into hemispheres, ipsilateral and contralateral to the MCAO, and we analyzed each hemisphere separately for PDGFR-α activation. After stroke, PDGFR-α phosphorylation was induced approximately twofold in the ipsilateral hemisphere of wild-type mice compared to that in the nonischemic hemisphere, whereas in tPA-deficient mice, no induction of PDGFR-α activation was seen (**Fig. 5a,b**). These data suggest that PDGFR-α activation within the brain after ischemic stroke is tPA dependent through PDGF-CC activation.

To test whether MCAO-induced activation of the PDGFR-α regulates the cerebrovascular response to stroke, we treated mice with the PDGFR-α inhibitor[32] imatinib mesylate 1 h after MCAO. Mice treated with imatinib showed a 33% reduction in Evans blue extravasation after MCAO compared to control mice (**Fig. 5c–e**), suggesting that PDGFR-α signaling regulates cerebrovascular permeability after stroke. To determine whether this effect was due to activation of PDGFR-α by PDGF-CC, we injected PDGF-CC–specific antibodies intraventricularly into mice immediately before stroke induction and evaluated Evans blue extravasation 24 h after MCAO. These data show that, like imatinib treatment, Evans blue extravasation was significantly ($P < 0.05$) reduced in mice treated with PDGF-CC–specific antibodies but not in mice receiving preimmune IgG (**Fig. 5f**). This supports the hypothesis that endogenous PDGF-CC mediates the regulation of cerebrovascular permeability after stroke via activation of PDGFR-α.

Because reducing the extent of BBB dysfunction during stroke may affect infarct expansion and improve stroke outcome, we compared infarct volumes in mice treated with imatinib to control mice 72 h after MCAO. Imatinib significantly ($P < 0.05$) reduced lesion volume by 34% (**Fig. 5g**), suggesting that interfering with the PDGF-CC–PDGFR-α system may improve stroke outcome.

A major risk associated with the use of thrombolytic tPA in ischemic stroke is the occurrence of hemorrhagic complications[33–36]. Because bleeding represents an extreme example of loss of BBB integrity, we examined whether inhibition of PDGFR-α with imatinib administered 1 h after the onset of ischemia could reduce the extent of hemorrhagic complications associated with tPA-mediated thrombolysis initiated 5 h after the induction of MCAO. These experiments

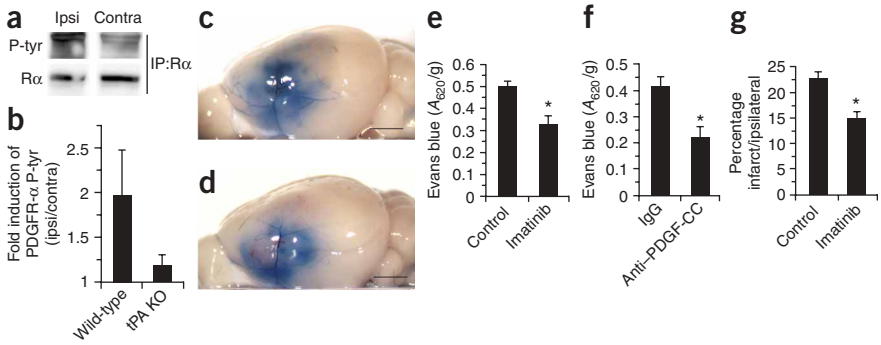

Figure 5 Blocking PDGFR-α activation reduces cerebrovascular permeability and stroke volume after MCAO. (**a**) PDGFR-α activation after MCAO in wild-type mice 6 h after MCAO. Brains were divided into ipsilateral and contralateral hemispheres and detergent-solubilized. The lysates were immunoprecipitated with PDGFR-α–specific antibodies followed by immunoblotting with phosphotyrosine-specific antibodies (P-tyr). As a loading control, the precipitated protein was visualized with PDGFR-α–specific antibodies (Rα). (**b**) Quantification of PDGFR-α activation after MCAO in wild-type or tPA KO mice ($n = 2$ in each group and data represent means ± s.e.m.). The standard error of the mean was calculated from the sample standard deviation divided by the square root of the sample size. (**c**,**d**) Representative images of the ipsilateral hemisphere 24 h after MCAO from control (**c**) and imatinib-treated (**d**) mice injected with Evans blue 1 h before euthanasia. (**e**) Quantification of the Evans blue extravasation 24 h after MCAO ($n = 11$ for each group and data represent means ± s.e.m.). (**f**) Quantification of the Evans blue extravasation 24 h after MCAO in mice treated with preimmune IgG or PDGF-CC–specific antibodies followed immediately by MCAO ($n = 10$ for each group and data represent means ± s.e.m.). (**g**) Quantification of infarct size 72 h after MCAO in mice treated with either imatinib or vehicle ($n = 9$ for control group and $n = 11$ for imatinib group; data represent means ± s.e.m.). For all imatinib studies, mice were treated by gavage with either vehicle or 200 mg/kg imatinib 1 h and 8 h after MCAO and twice daily for the duration of the experiment. *$P < 0.05$ versus control mice. Scale bars, 2 mm.

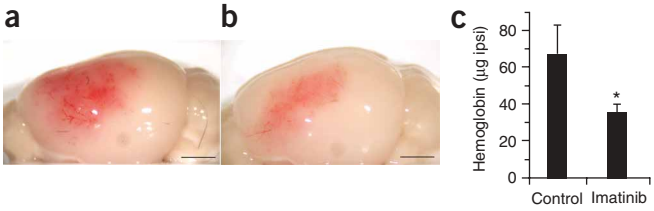

Figure 6 Blocking the PDGF-CC–PDGFR-α pathway reduces intracerebral hemorrhage after MCAO. (**a**,**b**) Representative images of the intracerebral hemorrhage 24 h after MCAO in the ipsilateral hemisphere of mice treated with tPA 5 h after MCAO. Mice were treated by gavage with either vehicle (**a**) or 200 mg/kg imatinib (**b**) 1 h and 8 h after MCAO. (**c**) Quantification of hemoglobin content in the ischemic hemisphere 24 h after MCAO ($n = 10$ for control group and $n = 12$ for imatinib group; data represent means ± s.e.m.). *$P < 0.05$ versus control mice. Scale bar, 2 mm.

indicated that imatinib treatment significantly ($P < 0.05$) decreased hemorrhage, reducing the amount of hemoglobin associated with the ischemic hemisphere by 50% compared to that in untreated mice (**Fig. 6a–c**). This suggests that the known association of thrombolytic tPA with hemorrhagic complications in some patients may be due in part to the activation of PDGF-CC by therapeutic tPA.

DISCUSSION

Thrombolytic treatment benefits only a limited number of patients with ischemic stroke, and the development of improved therapies for stroke depends upon understanding the unique characteristics of the cerebrovasculature. The limitations of tPA seem to be due in part to unique activities that tPA has in the brain beyond its well established role in fibrinolysis[9,17,19,37,38]. Although there are clear benefits to people who receive early thrombolytic treatment[3,15], the nonfibrinolytic effects of tPA suggest that there are unique challenges for the use of thrombolytic therapy in ischemic stroke. To understand these challenges, it is necessary to understand the role that endogenous tPA has in the CNS. It is clear that immediately after stroke onset, ischemia induces changes in cerebrovascular structures. We suggest that these changes include the release of tPA into perivascular tissue[17]. This release precedes the loss of BBB integrity and seems to be mechanistically essential for the early loss of BBB function, as mice lacking tPA are protected from the loss of barrier function[17]. The downstream target of tPA in this system is not plasminogen, as mice lacking plasminogen show a similar degree of cerebrovascular permeability to wild-type mice[17]. Other potential CNS targets include the *N*-methyl-D-aspartic acid receptor and LRP[9,24,39]. Experiments using antagonists of these receptors suggest that the *N*-methyl-D-aspartic acid receptor is not directly involved, whereas antagonists of LRP reduce cerebrovascular permeability after MCAO[17,24]. Our studies support this hypothesis and also identify PDGF-CC as a specific substrate of tPA within the neurovascular unit. PDGF-CC is a member of the PDGF family that binds the PDGFR-α. Unlike PDGF-A or PDGF-B, PDGF-C contains an N-terminal CUB domain that renders the dimer inactive. However, removal of the CUB domains by tPA[20] generates active PDGF-CC, which can then activate the PDGFR-α in the neurovascular unit.

The direct injection of active PDGF-CC into the CSF bypasses the requirement for activation and increases cerebrovascular permeability in nonischemic brain. In contrast to tPA-induced vascular permeability[17], the enhanced permeability of active PDGF-CC is independent of LRP, implying that both tPA and LRP act upstream of active PDGF-CC. Consistent with this, we show that cell-associated LRP enhances tPA-mediated activation of PDGF-CC. In the context of the neurovascular unit, LRP may be a necessary cofactor for efficient activation of latent PDGF-CC by tPA. We also show that PDGF-CC and PDGFR-α are expressed in cerebral arterioles, as is tPA[40]. The localization of tPA, LRP, PDGF-CC and the PDGFR-α within the neurovascular unit of arterioles may facilitate the response to ischemia through activation of the PDGFR-α.

Electron microscopy demonstrates that the development of edema in response to tPA or PDGF-CC is not due to gross destruction of vascular structures or the basement membrane. Instead, these data suggest that the development of edema may be occurring through a regulated process and thus may represent an exaggeration of a normal vascular response. In pathological conditions such as stroke, neuronal depolarization associated with cerebral ischemia can result in a surge of local tPA activity[17,41], which in turn could lead to continued production of active PDGF-CC, persistent activation of PDGFR-α in the neurovascular unit and ultimately loss of BBB integrity.

The benefit of thrombolytic tPA administered within the first 3 h of stroke is probably dependent upon the early maintenance of BBB integrity. In this situation, thrombolysis of the occluded vessel should rescue the affected ischemic zone and improve clinical outcome. However, administering tPA beyond the 3 h window increases the likelihood that changes in the cerebrovasculature impair BBB integrity to the point that thrombolytic tPA crosses into the perivascular tissue[16] and interacts with the neurovascular unit. We postulate that this tPA may further increase activation of endogenous PDGF-CC, thus prolonging activation of the PDGFR-α. This leads to further deterioration of BBB integrity and in extreme cases may lead directly to hemorrhagic complications.

Understanding this mechanism offers an opportunity to potentially extend the treatment window of tPA for stroke patients. Because blocking the PDGF-CC–PDGFR-α pathway is unlikely to disrupt tPA's fibrinolytic function, strategies specifically targeting the PDGF-CC–PDGFR-α pathway should maintain tPA's beneficial thrombolytic activity while minimizing BBB dysfunction. Imatinib, also known as Gleevec or STI571, is a US Food and Drug Administration–approved drug for the treatment of chronic myelogenous leukemia and other cancers that binds and inhibits several tyrosine kinases, including PDGFR-α. In our study, imatinib treatment reduced cerebrovascular permeability and stroke lesion volume as well as hemorrhagic complications associated with late thrombolysis, suggesting the possibility of an off-the-shelf adjunct therapy for use with thrombolytic tPA. Although imatinib does not efficiently cross the BBB in healthy individuals, imatinib is present in the CNS after oral administration[42]. In addition, dose and time requirements for using imatinib in stroke should be very different than in antitumor applications. A transient high dose of imatinib may be all that is required to extend the therapeutic window for tPA. However, further studies are needed to explore this possibility, as it remains to be determined whether late administration of a combination therapy of imatinib plus tPA can extend the standard 3 h treatment window of tPA by reducing hemorrhagic complications and restoring neuroprotection. Toward this goal, a randomized controlled clinical trial is currently planned by the stroke research team at Karolinska University Hospital in Stockholm, Sweden (N. Wahlgren, Karolinska University Hospital, personal communication). This trial is designed to evaluate the safety and feasibility of imatinib, either alone or with tPA, for use within the first few hours after the onset of ischemic stroke.

In summary, our data offer a new model for regulation of cerebrovascular permeability by endogenous tPA and PDGF-CC and suggest a potential new interventional approach that may extend tPA's

therapeutic window in ischemic stroke. Future studies may also examine whether other CNS activities affected by tPA, such as seizure spreading, susceptibility to drug addiction or anxiety-like behaviors[43], also involve tPA-mediated activation of PDGF-CC.

METHODS

Proteins and antibodies. We produced the recombinant core domain of human PDGF-CC in baculovirus-infected insect Sf9 cells and purified it as previously described[21]. Blocking antibodies to active PDGF-CC (**Supplementary Fig. 2**) were affinity-purified rabbit IgG isolated from rabbit 615 as previously described[21]. Preimmune IgG from the same rabbit was isolated on a protein A–Sepharose column (Amersham Biosciences). Mouse tPA and affinity-purified antibodies to mouse tPA were from Molecular Innovations.

Cell culture and immunoblotting. We maintained all cells used in DMEM supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. We cultured cells at 37 °C in a humidified 5% CO_2 atmosphere. We obtained the mouse astrocyte cell line C8-D1A from the American Type Culture Collection and grew it in the presence of 1 mM pyruvate. All cell culture reagents were from Gibco. We kept subconfluent cultures of wild-type and *Lrp1*$^{-/-}$ MEF cell lines[25] in serum-free DMEM overnight with or without the addition of 1 μg/ml tPA. We collected conditioned serum-free medium and precipitated proteins with trichloroacetic acid as previously described[21]. We detected PDGF-C species by immunoblotting with PDGF-CC–specific antibodies (from rabbit 615).

Intraventricular injections. To perform intraventricular injection of tPA, active PDGF-CC and PDGF-CC–specific antibodies, we anesthetized wild-type C57BL/6J mice with chloral hydrate (450 mg/kg, intraperitoneally), placed them on a stereotactic frame and injected them at bregma –2, mediolateral 0, and dorsoventral 2 (ref. 17). Injections contained 3 μl of either active tPA (3 μM), active PDGF-CC core protein (3 μM), PDGF-CC–specific antibodies (0.4 mg/ml), preimmune IgG (0.4 mg/ml) or PBS. We then determined cerebrovascular permeability 1 h later by Evans blue extravasation as described in **Supplementary Methods** online, except that brains were not divided into hemispheres. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Unit for Laboratory Animal Medicine at University of Michigan.

Electron microscopic analysis. One hour after intraventricular injection of either PBS, tPA or active PDGF-CC, we perfused C57BL/6J mice with PBS followed by 4% formaldehyde with 1% glutaraldehyde and fixed the tissue with the same solution for 24 h. We then rinsed the brain tissues with 5% sucrose, 0.1 M phosphate buffer pH 7.2 three times over 30 min, post-fixed the tissues in 1% aqueous OsO_4 for 1 h and then stained with 1% uranyl acetate (Electron Microscopy Science) for 1 h. After dehydration, we embedded the brain tissues in EPON (EMS) and cut and stained ultra thin sections with uranyl acetate and lead citrate. We stained some sections with toluene blue for analysis by light microscopy. For electron microscopic analysis, we examined sections with a Phillips CM12 transmission electron microscope.

Immunohistochemical localization. For immunohistochemistry, we perfused mice with 4% paraformaldehyde in PBS, post-fixed the brains overnight in the same fixative, processed them for paraffin-embedding and then sectioned them to 6 μm by standard protocols. We performed immunohistochemical staining of PDGF-CC and tPA expression with affinity-purified rabbit IgG against human core PDGF-CC (10 μg IgG/ml) and affinity-purified rabbit antibody to mouse tPA (15 μg IgG/ml), respectively. Controls with normal rabbit IgG gave only background staining (data not shown). We used an Elite ABC Vectastain kit (Vector Laboratories). We performed blocking of nonspecific binding with TNB blocking buffer (TSA indirect NEN Bioscience). For the tPA staining, we performed antigen retrieval with 0.1 M citrate buffer pH 3.0 at 95 °C for 20 min followed by treatment with 0.25% trypsin at 37 °C for 20 min before the immunostaining as described above.

Immunofluorescence localization. Details of the immunofluorescence localizations are presented in **Supplementary Methods**.

Analysis of receptor activation. We determined PDGFR-α activation essentially as previously described[44]. Briefly, for cell culture studies, we stimulated subconfluent astrocyte cultures with 100 ng/ml active PDGF-CC for 90 min on ice or treated them with tPA overnight and then lysed them as previously described[21]. We subjected cell lysates directly to immunoblotting and determined receptor tyrosine phosphorylation with a phosphotyrosine-specific antibody (PY99, Santa Cruz). As a loading control, we also performed immunoblotting of the endoplasmic reticulum integral membrane protein calnexin (sc-6465, Santa Cruz). For analysis of receptor activation after MCAO, we subjected both wild-type and tPA knockout mice (10-week-old males) to MCAO. Six hours later, we perfused mice with PBS for 4 min and removed the brains and separated them into two hemispheres. We then processed the ipsilateral and contralateral hemispheres for PDGFR-α activation by immunoprecipitation of the PDGFR-α with a specific PDGFR-α antibody followed by immunoblotting for receptor tyrosine phosphorylation with the same phosphotyrosine-specific antibody as in the cell culture studies above. We monitored the amount of precipitated PDGFR-α by reprobing the blots with a PDGFR-α–specific antibody (R&D Systems).

Mouse model of ischemic stroke. Details of the mouse model of ischemic stroke are presented in **Supplementary Methods** and **Supplementary Figure 6**.

Hemoglobin content assay. Twenty four hours after MCAO, we anesthetized mice with chloral hydrate (450 mg/kg intraperitoneally, Morton Grove Pharmaceuticals) and killed them by exsanguination. We then perfused the mice with PBS for 4 min, removed the brains and separated them into hemispheres ipsilateral and contralateral to the MCAO. We homogenized each hemisphere (Model T8, IKA Works) in 475 μl PBS at 25,000 r.p.m. for 30 s on ice in a 1.5-ml microfuge tube. We added 25 μl 10% Triton X-100 (Fisher Biotech) to the tube to make the final concentration 0.5%. After thorough mixing, we kept the tube at 23 °C for 5 min and then centrifuged at 25,000*g* at 4 °C for 30 min (Eppendorf 5417R). We read 50 μl of the supernatant at $A = 410$ nm (SpectraMax M5, Molecular Devices) and quantified the hemoglobin content relative to a purified hemoglobin (Sigma-Aldrich) standard curve as previously described[45].

Statistical analyses. We analyzed data by either a Wilcoxon Mann rank-sum test or, in cases where more than one group is compared, by ANOVA with repeated test (GraphPad, InStat). *P* values smaller than 0.05 were considered significant.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

E.J.S. and L.F. conceived, designed and performed the research and wrote the paper, M.G., E.F., J.C., J.A., Y.G., K.P. and K.M. performed the research, M.Y. conceived the research, D.K.S. and C.B. designed the research and contributed vital reagents, U.E. and D.A.L. conceived and designed the research and wrote the paper.

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