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第3次対がん総合戦略研究事業

血管新生とリンパ管新生の同時制御による制癌法の確立に関する研究

平成21年度 総括・分担研究報告書

研究代表者 佐藤 靖史

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目　　次

厚生労働科学研究費補助金（第３次対がん総合戦略研究事業）
総括研究報告書

血管新生とリンパ管新生の同時制御による制癌法の確立に関する研究

研究代表者　佐藤靖史　東北大学加齢医学研究所教授

研究要旨
大腸菌を用いて、活性を保持したレコンビナントVasohibin-1蛋白の調整法を確立した。腹腔内などVASH1の局所療法の有用性を提示した。Vasohibinファミリーのもう１つの分子であるVasohibin-2について、VASH-1と拮抗して血管新生を促進すること、しかもVasohibin-2はがん細胞に発現し、腫瘍血管新生を促進して腫瘍発育に寄与していることを明らかにし、Vasohibin-2が、がん治療の新たな分子標的になることを示した。

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A．研究目的

癌の治療標的として腫瘍血管新生と腫瘍リンパ管新生が注目されている。理想としては血管新生とリンパ管新生の双方を同時に制御することが望ましいが、そのような治療法は未だ確立していない。本研究は、癌治療へのVasohibinファミリー分子の解析を進めることで、本邦発の新しい治療法として国民の癌医療に大きく貢献することを目的としている。

B．研究方法

(1) 大腸菌を用いたレコンビナントVasohibin-1蛋白の調整法について、精製方法やリフォールディングなどの条件を検討する。
(2) 精製されたVasohibin-1の抗腫瘍活性を、マウスモデルを用いて検証する。
(3) Vasohibinファミリーのもう１つの分子であるVasohibin-2のがん組織における局在を検証する。
(4) がんにおけるVasohibin-2の意義を、動物モデルを用いて検証する。
（倫理面への配慮）
全ての動物実験は所属施設での審査を受けた後に行う。

C．研究結果

(1) 内皮細胞を障害せずに、広いスペクトルムで血管新生とリンパ管新生を抑制するVasohibin-1について、大腸菌由来組換え蛋白のリフォールディングによる分散画分（活性画分）調製法の改良を進め、活性を有する組換え蛋白の調整法を確立した。
(2) 動物モデルにおいて、この組換えVasohibin-1蛋白を腫瘍局所に注射すると抗腫瘍効果は得られるが、血中での安定化を目的にPEG化修飾した蛋白の全身投与では、PEG化によって活性化が低下するため十分な抗腫瘍効果は得られなかった。一方、腹膜播種すると腹水を形成して急速に個体を死に至らしめるヒト卵巣癌由来SKOV-3細胞にVasohibin-1遺伝子を安定導入すると、mockコントロールではヌードマウスの腹腔内移植で4週間以内に全て死亡するのに対し、Vasohibin-1遺伝子導入群では腹水も生じず、全て10週間以上生存するという顕著な効果を観察した。
(3) Vasohibin-1ホモログのVasohibin-2は、血管新生局所に浸潤する骨髄由来単核球に発現し、Vasohibin-1と拮抗して血管新生を促進することが判明しているが、癌細胞の一部にも発現することが判明した。そこでVasohibin-2発現陰性の腫瘍細胞にVasohibin-2を導入してマウスに移植すると、腫瘍血管新生と腫瘍発育が増強することが判明した。

D．考察

レコンビナントVasohibin-1蛋白の調整法は確立できたが、血管をルートとして全身投与する治療法への応用は、現時点では未だ困難である。しかし、腹腔内投与など、局所療法への応用は、可能性の高い手法として、今後検討したい。

今年度の最も大きな収獲は、がんにおけるVasohibin-2の意義が明らかになったことである。Vasohibin-2はがん細胞に発現し、腫瘍血管新生を促進して腫瘍発育に寄与している。よって、Vasohibin-1を外因性に投与すると同時にVasohibin-2の

作用を阻害することが、がん治療における望ましい治療法として提唱したい。

E．結論

レコンビナント Vasohibin-1 蛋白の大量調整法を確立すると共に、がんにおける Vasohibin-2 の意義が明らかにし、Vasohibin-2 が、がん治療の新たな分子標的となえう可能性を示した。がん治療においては、外因性の Vasohibin-1 蛋白を投与に Vasohibin-2 の作用阻害を併用することの有用性が示唆された。

F．健康危険情報

健康に対する危険性を認めない。

G．研究発表

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高橋詳史，中村崇宣，小林美穂，鈴木康弘，宮下浩輝，佐藤靖史. Vasohibin-2 Expressed in Tumors Accelerates Tumor Growth by Promoying Angiogenesis. The 1st International Global COE Symposium，仙台，2009.

H．知的財産権の出願・登録状況

1．特許

佐藤靖史　固形癌の存在の検出法　特許出願 2009-190830

佐藤靖史、近藤丘　抗バゾヒビンモノクローナル抗体のセット　特許出願 PCT/JP2009/64862

佐藤靖史　血管新生促進因子　特許出願 PCT/JP2009/051359

2．実用新案登録

なし

3. その他

なし

厚生労働科学研究費補助金（第３次対がん総合戦略研究事業）
（総括・分担）研究報告書

骨髄細胞・内皮前駆細胞に関する研究に関する研究

研究分担者　高倉　伸幸　大阪大学微生物病研究所教授

研究要旨：vasohibin-1は血管新生抑制因子として、抗腫瘍血管新生にむけた臨床応用に期待されている。腫瘍内の血管に特異的にvasohibin-1をデリバリーする方法論を得るため、腫瘍内皮細胞に特異的に発現する分子の単離を行い、TSR(仮名)を同定した。

A．研究目的

血管新生抑制因子として単離されたvasohibin-1(以下vash1)を腫瘍内の内皮細胞に特異的かつ大量に運搬するためには腫瘍の内皮細胞に特異的に発現する分子を単離し、これら分子をターゲットとして薬剤を導入する必要がある。本研究では、腫瘍内の内皮細胞に特異的に発現する分子の単離を試みた。

B．研究方法

低酸素あるいはVEGFで発現が亢進する分子をリストアップし、腫瘍内の内皮細胞特異的発現性を解析した。

（倫理面への配慮）

本研究はマウスを用いた解析であり、実験動物の使用に関しては、大阪大学微生物病研究所の定める倫理規定に則って研究を行った。

C．研究結果

本研究においてほぼ腫瘍領域全般に内皮細胞全体に発現するTSRを単離した。本TSRは内皮細胞膜に発現する受容体であり、その結合因子TSRLも同じく腫瘍内の内皮細胞に同時に発現が観察された。TSRは従来より、血管内皮細胞成長因子（VEGF）によって発現が誘導され、血管新生時に血管拡大に機能することを報告してきたが、血管透過性の抑制効果があることも解明された。

D．考察

TSRに対する抗体、あるいはTSRLを利用して腫瘍内の血管内皮細胞にvash1の導入が可能であると考えられる。PEG化リポソームの表面にTSRLをコーティングしたDDS法を構築しており、本法によるvash1のデリバリーに向け検討を継続する。

E．結論

TSRは腫瘍内の血管内皮細胞特異性が高く腫瘍特異的DDSに応用可能と考えられた。

F．健康危険情報

G．研究発表

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H. 知的財産権の出願・登録状況
（予定を含む。）
1. 特許取得
なし
2. 実用新案登録
なし
3. その他
なし

厚生労働科学研究費補助金（第３次対がん総合戦略研究事業）
分担研究報告書

リンパ管内皮細胞の遺伝子発現に関する研究

分担研究者　渡部　徹郎　東京大学大学院医学系研究科・准教授

研究要旨　癌の悪性化を抑制するためには血管新生とリンパ管新生の両方を同時に制御できる治療法を開発することが急務であり、内因性の血管・リンパ管新生抑制因子である Vasohibin-1 に注目が集まっている。本研究では Vasohibin ならびにさまざまな調節因子による血管・リンパ管新生抑制作用の分子機構の解明を試みた。本年度は主にリンパ管新生を調節する Ets-2 ならびに HoxD8 転写因子の機能についての研究を行い、Ets-2 の機能を阻害することによってリンパ管新生が阻害されることを見出した。

A．研究目的

リンパ管は末梢組織で血管から漏出した間質液、タンパク質、細胞などを血管系へと環流することにより血液の量や組成を一定に保ち、成体の恒常性を維持するとともに、癌組織から細胞を運んでリンパ節転移を引き起こしたりもする。このためリンパ管形成機構の解明は医学的に必要性が高いが、その研究の歴史は比較的新しく、未解明な点が多く残されている。本研究においてはVasohibinならびに他の調節因子によるリンパ管新生抑制作用の分子機構の解明を試みるために、本年度は血管新生において重要な役割を果たすEts-2とHoxD8転写因子に注目し、リンパ管新生における作用について検討した。

B．研究方法

我々はヒト臍帯静脈内皮細胞(HUVEC)ならびにヒト皮膚由来リンパ管内皮細胞(HDLEC)を用いてEts-1の作用を検討した。HUVECならびにHDLECにおいてEts-2ならびにHoxD8の発現を増減させるためにそれぞれアデノウィルスとsiRNAを用いた。さらに個体レベルで両者のリンパ管新生に対する作用を検討するためにマウス横隔膜上の新生リンパ管において腹腔内に注射したアデノウィルスによりそれぞれの転写因子を発現させた。

（倫理面への配慮）

ヒトの遺伝子解析ならびに相手方の同意を得る研究は本研究計画には含まれていない。

C．研究結果

我々は昨年度Ets-1がリンパ管新生を誘導することを報告したが、Ets-2の発現を低下させてもリンパ管新生は抑制されなかった。しかしEts-2の発現を低下させるとリンパ管新生に重要な役割を果たすPDGF受容体(PDGFRβ)の発現が低下し、リンパ管新生が抑制された。さらにマウス個体レベルでEts-2の機能を阻害するTm-Ets変異体を発現させることによりリンパ管新生が抑制されることが確認された（Yoshimatsu, Watabe et al.,投稿準備中)。

さらにProx1の標的遺伝子としてHoxD8転写因子を同定し、HoxD8がProx1の発現を亢進するポジティブフィードバック因子であることを明らかにした。さらにHoxD8をマウス個体のリンパ管において発現するとその管径が増大することを見出した(Harada, Watabe et al., 2009, Journal of Cell Science)。

D．考察

以上の結果からEts-2ならびにHoxD8がリンパ管新生を抑制するための新たな標的となりうることが示唆された。

E．結論

Ets-2とHoxD8はリンパ管内皮細胞においてそれぞれProx1の機能と発現を調節することによりVEGFR3などのリンパ管新生シグナル伝達因子の発現調節を行ない、リンパ管新生を誘導していることが明らかになった。

F．健康危険情報

該当なし

G．研究発表

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Watabe T, Kokudo T, Suzuki Y, Yoshimatsu Y, Yamazaki T, Miyazono K. Snail is required for TGF-β-induced endothelial-mesenchymal transition of embryonic stem cell-derived endothelial cells (Symposium)

第 32 回日本分子生物学会年会（横浜）2009 年 12 月 9～12 日
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H．知的財産権の出願・登録状況

1．特許取得
特になし
2．実用新案登録
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3.その他
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研究成果の刊行に関する一覧表レイアウト

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ

雑誌

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Effect of Recombinant Placental Growth Factor 2 on Hypertension Induced by Full-Length Mouse Soluble fms-Like Tyrosine Kinase 1 Adenoviral Vector in Pregnant Mice

Hirotada Suzuki, Akihide Ohkuchi, Shigeki Matsubara, Yuji Takei, Masato Murakami, Masabumi Shibuya, Mitsuaki Suzuki and Yasufumi Sato

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Abstract—The first aim of our study was to develop a pregnant mouse model for preeclampsia using adenoviral vector containing mouse full-length soluble fms-like tyrosine kinase 1 (sFlt-1) but not truncated sFlt-1. The second aim was to evaluate effects of recombinant mouse (rm) vascular endothelial growth factor (VEGF) and rm placental growth factor (PlGF) on a preeclampsia model induced by adenoviral vector containing mouse full-length sFlt-1. We injected adenoviral vector containing mouse full-length sFlt-1 on day 8.5 or 9.5 of gestation into pregnant Institute of Cancer Research mice, resulting in hypertension, proteinuria, and similar glomerular histological changes as those seen in human preeclamptic women with glomerular endotheliosis on day 16.5 or 17.5 of gestation. The preeclampsia models were treated with 100 μg/kg of rmVEGF164 (n=5), 100 μg/kg of rmPlGF-2 (n=5), or vehicle (n=7) twice a day for 2 days IP. The rmVEGF164 treatment significantly decreased the mean blood pressure on day 16.5 or 17.5 of gestation compared with the vehicle treatment (85±4 versus 97±2 mm Hg; P=0.018). The rmPlGF-2 treatment also significantly decreased the mean blood pressure on day 16.5 or 17.5 of gestation compared with the vehicle treatment (86±3 versus 97±2 mm Hg; P=0.018). However, proteinuria was not affected by either rmVEGF164 or rmPlGF-2. In conclusion, we, for the first time, created a mouse preeclampsia model using mouse full-length sFlt-1. VEGF and PlGF may be promising for ameliorating hypertension in women with preeclampsia. Additional study of PlGF as a potential drug for preeclampsia is warranted. (***Hypertension.* 2009;54:1129-1135.**)

Key Words: adenoviral vector ■ soluble fms-like tyrosine kinase 1 ■ vascular endothelial growth factor ■ placental growth factor ■ preeclampsia ■ animal models ■ therapy

Preeclampsia is associated with maternal and infantile morbidity and mortality.[1,2] It has been shown that the concentration of soluble fms-like tyrosine kinase 1 (sFlt-1), a circulating antiangiogenic protein, is increased in women with preeclampsia,[3,4] and increased levels of sFlt-1 and reduced levels of free placental growth factor (PlGF) are potentially useful for predicting the subsequent development of preeclampsia.[4,5] SFlt-1 acts by adhering to the receptor-binding domains of vascular endothelial growth factor (VEGF)-A and PlGF, preventing their interaction with endothelial receptors on the cell surface. Recent studies have indicated that patients with cancer receiving anti-VEGF antibody therapy may have an increased incidence of proteinuria and hypertension because of a decrease in their circulating VEGF levels.[6] Nonpregnant and pregnant rodents administered anti-VEGF antibodies or sFlt-1 manifested proteinuria and hypertension.[3,7,8] These results strongly indicate that increases in sFlt-1 and decreases in VEGF/PlGF in the maternal circulation may cause the occurrence of preeclampsia.

SFlt-1, a human natural soluble form of the VEGF receptor (VEGFR) 1, is produced in conditioned culture medium of human umbilical vein endothelial cells[9] and in the trophoblasts.[10,11] SFlt-1 is encoded by the flt-1 gene and is truncated between N-terminal immunoglobulin-like domains 6 and 7.[12] Because the N-terminal first and second domains of Flt-1 are necessary and sufficient for the binding of VEGF-A with near-native affinity,[13,14] truncated sFlt-1$^{1-3}$ containing the first to third domains, but not full-length sFlt-1, has been used for the studies evaluating the effect of sFlt-1 on hypertension and proteinuria in mouse or rat models.[3,7,15–19] However, there are 2 differences between the full-length sFlt-1 and truncated sFlt-1$^{1-3}$: first, the full-length sFlt-1 has a 31-amino acid carboxyl lesion derived from an intron, which is significantly homologous to that in mammals,[11] and, second, the truncated sFlt-1$^{1-3}$ lacks the immunoglobulin-like loop 4, which is essential to stabilize receptor dimerization of the extracellular domains of Flt-1, in addition to VEGF.[13,14] Therefore, the effect of sFlt-1 on the occurrence of hyperten-

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sion or proteinuria may be different between truncated sFlt-1 and natural full-length sFlt-1.

It has been reported that the effect of excess circulating sFlt-1 can be ameliorated by the administration of recombinant VEGF-A.[7,19] However, in humans, a decrease in PlGF is related to the later occurrence of preeclampsia.[20–22] Therefore, we also hypothesized that not only VEGF-A, but also PlGF, may play a pivotal role in the occurrence of hypertension and proteinuria in both humans and rodents. To our knowledge, the effect of the administration of PlGF into a rodent model of preeclampsia via a mouse (m)-sFlt-1 adenoviral vector has not been examined.

First, we evaluated an adenovirus encoding the full-length mouse-sFlt-1 gene (Ad m-sFlt-1) for the induction of hypertension and proteinuria in pregnant mice. Second, we evaluated the effects of recombinant mouse (rm) VEGF164 (rmVEGF164) and rmPlGF-2 on hypertension and proteinuria in a mouse preeclampsia model induced by Ad m-sFlt-1.

Methods

An expanded Materials and Methods section is available in the online Data Supplement (available at http://hyper.ahajounals.org). Briefly, in the first experiment, an Ad m-sFlt-1, which was created in our previous study,[23] and an adenovirus encoding β-galactosidase gene (Ad LacZ) were propagated in HEK293cells. The viral lysates were purified and concentrated through 2 cycles of CsCl step gradients.[24] Nine- to 12-week–old CD1 (Institute of Cancer Research) mice were injected in the tail vein with 3×10^8 plaque-forming unit (PFUs; low dose), 1×10^9 PFUs (medium dose), or 2×10^9 PFUs (high dose) of Ad m-sFlt-1 (n=9, n=6, and n=6, respectively) and with 3×10^8 PFUs (low dose), 1×10^9 PFUs (medium dose), or 2×10^9 PFUs (high dose) of Ad LacZ (n=7, n=6, and n=7, respectively) on day 8.5 or 9.5 of gestation. The control pregnancy mice were not injected with anything (n=7). The mean blood pressures (MBPs) were measured by the tail-cuff method (Softron Ltd) on 4 different days: (1) before mating, (2) just before the injection of adenovirus, (3) on day 13.5 or 14.5 of gestation, and (4) on day 16.5 or 17.5 of gestation. The urine albumin:creatinine (Alb/Cre) ratios on day 16.5 or 17.5 of gestation were measured. In the second experiment, pregnant mice were injected in their tail vein with 2×10^9 PFUs (high dose) of Ad m-sFlt-1 on day 8.5 or 9.5 of gestation. rmVEGF164 (100 μg/kg diluted in 500 μL of PBS; n=5) was administered IP twice a day for 2 days from the evening on day 14.5 or 15.5 of gestation. In other mice, rmPlGF-2 (100 μg/kg diluted in 500 μL of PBS; n=5) and the vehicle (500 μL of PBS; n=7) were administered IP twice a day for 2 days. The MBP and Alb/Cre ratio were also measured. All of the animal housing and experiments were approved by the institutional animal care and research advisory committee of both the University of Tohoku and Jichi Medical University. The pharmacokinetics of rmVEGF164 and rmPlGF-2 in nonpregnant mice and pregnant mice are shown in the online Data Supplement (available at http://hyper.ahajournals.org, Figure S1A through S1D).

Results

Expression of Proteins by the Adenoviral Vector

In mice administered Ad LacZ, β-galactosidase activity was observed in the liver but not in the placenta by 5-bromo-4-chloro-3-indolyl β-D-galactoside staining, suggesting that Ad m-sFlt-1 was expressed in the liver. The levels of mouse sFlt-1 (nanograms per milliliter) on day 16.5 or 17.5 of gestation increased significantly in the medium and high doses of Ad m-sFlt-1 compared with the medium and high doses of Ad LacZ, respectively (85 [58 to 95] versus, 12 [12 to 29], $P=0.002$; 93 [82 to 130] versus 25 [21 to 33], $P=0.001$, respectively; Figure 1A). The levels of mouse sFlt-1 on day 16.5 or 17.5 of gestation were not different among the control and the mice administered the low, medium, and high dose of Ad LacZ ($P=0.76$ by Kruskal-Wallis test).

Plasma Levels of Angiogenic Factors in Mice Administered Ad m-sFlt-1

The plasma levels of mouse VEGF-A (picograms per milliliter) on day 16.5 or 17.5 of gestation in the mice administered high-dose Ad m-sFlt-1 were significantly lower than in the mice administered high dose Ad LacZ (47 [43 to 52] versus 95 [93 to 113]; $P=0.001$; Figure 1B). On the contrary, the levels of mouse PlGF-2 (picograms per milliliter) on day 16.5 or 17.5 of gestation were almost the same among the control mice, the high-dose Ad LacZ group, and the high-dose Ad m-sFlt-1 group (21 [14 to 26], 25 [21 to 35], and 30 [23 to 38]; Figure 1C).

Blood Pressure and Proteinuria in Pregnant Mice Administered Ad m-sFlt-1

In the control mice and the mice administered low, medium, and high doses of Ad LacZ, MBP (millimeters of mercury) was almost the same during the prepregnancy period and during pregnancy (Figure 1D). In the mice administered high-dose Ad m-sFlt-1, MBP was significantly increased between day 8.5 or 9.5 and day 13.5 or 14.5 (76±2 versus 91±4; $P=0.028$) and between day 13.5 or 14.5 and day 16.5 or 17.5 (91±4 versus 101±3; $P=0.028$), although such increases were not seen in the mice administered low or medium doses of Ad m-sFlt-1 (Figure 1E). The MBP on day 16.5 or 17.5 of gestation in the high-dose Ad m-sFlt-1 group was significantly higher than that in the high-dose Ad LacZ group (101±3 versus 81±11; $P=0.010$) and the control group (83±4; $P=0.005$). The urine Alb/Cre ratios (milligrams per gram) on day 16.5 or 17.5 of gestation in the mice administered low, medium, and high doses of Ad m-sFlt-1 were significantly increased compared with those in the mice administered low, medium, and high doses of Ad LacZ, respectively (5.8 [4.5 to 14] versus 2.7 [2.4 to 2.9], $P=0.030$; 92 [43 to 148] versus 10 [4.9 to 22], $P=0.015$; 58 [30 to 161] versus 5.2 [4.0 to 13], $P=0.020$, respectively), although the urine Alb/Cre ratio in the low-dose Ad m-sFlt-1 group was not significantly different from that in the control mice (8.3 [3.6 to 11]; Figure 1F).

Histopathology in Mice Administered Ad m-sFlt-1

Glomerular histologies viewed by light microscopy in mice administered high-dose Ad m-sFlt-1 (Figure S2D and S2E) and those in the mice administered high-dose Ad LacZ (Figure S2A and S2B), scanning electron microscopy of glomerulus in mice administered high-dose Ad m-sFlt-1 and Ad Lac Z (Figure S2F and S2C), and the mean fetal and placental weights among the control mice, the high-dose Ad LacZ group, and the high-dose Ad m-sFlt-1 group (Figure S2G and S2H) were shown in the online Data Supplement. These results were written in the online Data Supplement.

Relationship Among the Plasma Levels of Mouse sFlt-1, Mouse VEGF-A, Blood Pressure, and Proteinuria

Among all of the data in control mice, mice administered Ad LacZ, and mice administered Ad m-sFlt-1, there was an

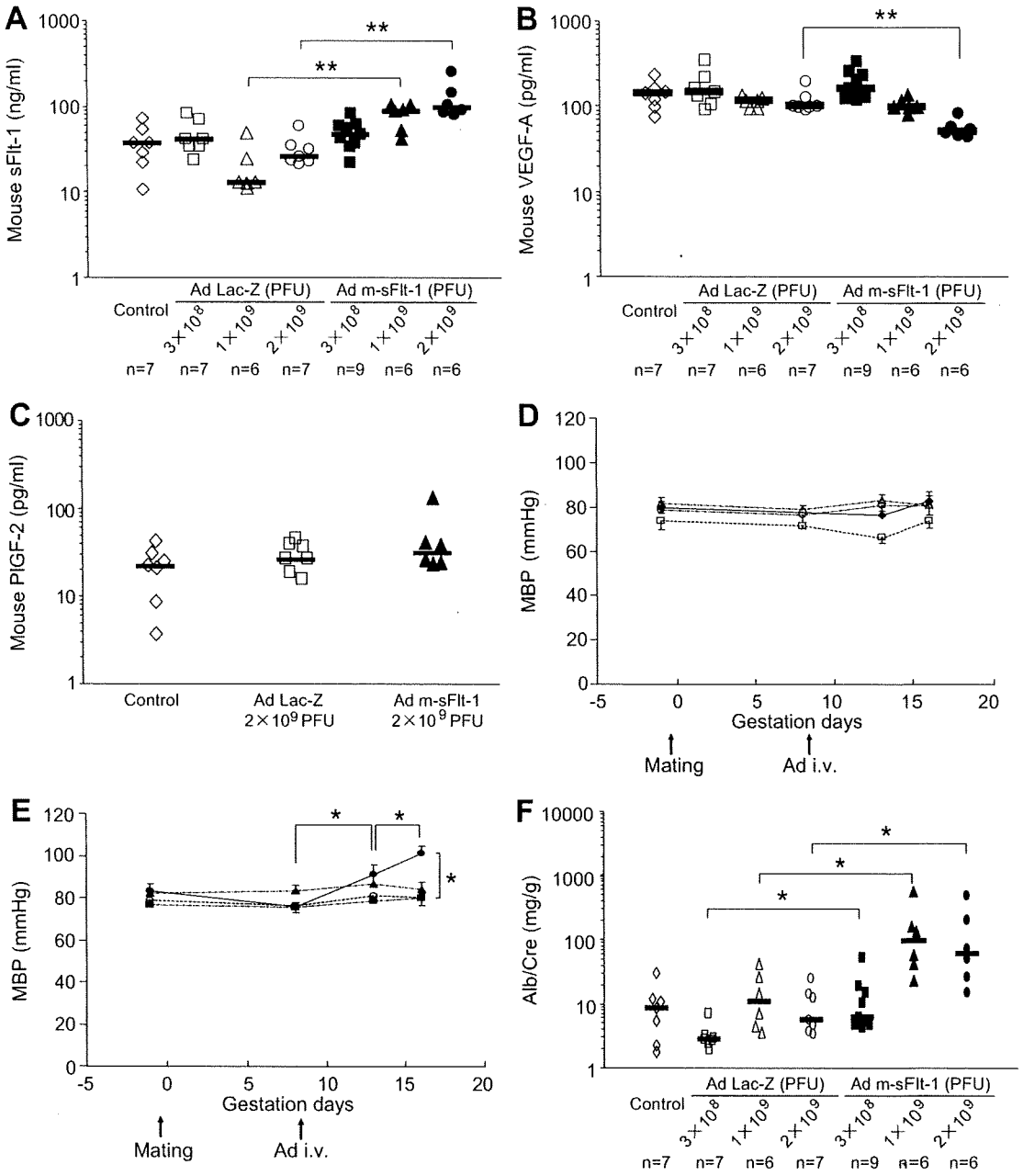

Figure 1. Data from pregnant mice administered nothing, Ad LacZ, and Ad m-sFlt-1. A, Plasma levels of m-sFlt-1 on day 16.5 or 17.5 of gestation. B, Plasma levels of m-VEGF-A (picograms per milliliter) on day 16.5 or 17.5 of gestation. C, Plasma levels of m-PlGF-2 (picograms per milliliter) on day 16.5 or 17.5 of gestation. D, MBP (millimeters of mercury) during the prepregnancy period, on day 8.5 or 9.5 of gestation, on day 13.5 or 14.5 of gestation, and on day 16.5 or 17.5 of gestation. ♦, □, △, and ○ represent the values in the control pregnant mice and pregnant mice administered low-dose (3×10^8 PFU), medium-dose (1×10^9 PFU), and high-dose (2×10^9 PFU) Ad LacZ, respectively. E, MBP (millimeters of mercury) during the prepregnancy period, on day 8.5 or 9.5 of gestation, on day 13.5 or 14.5 of gestation, and on day 16.5 or 17.5 of gestation. ○, ■, ▲, and ● represent the values in pregnant mice administered high-dose Ad LacZ and mice administered low-dose, medium-dose, and high-dose Ad m-sFlt-1, respectively. F, Urine Alb/Cre ratios (milligrams per gram) on day 16.5 or 17.5 of gestation. $^*P<0.05$; $^{**}P<0.01$.

inverse relationship between the plasma levels of log10sFlt-1 and log10VEGF-A ($r=-0.29$; $P=0.042$; Figure 2A); positive relationships between the plasma levels of log10sFlt-1 and MBP ($r=0.24$; $P=0.098$; Figure 2B) and between the plasma levels of log10sFlt-1 and urine log10(Alb/Cre; $r=0.44$; $P=0.002$; Figure 2C); and inverse relationships between the plasma levels of log10VEGF-A and MBP ($r=-0.33$; $P=0.023$; Figure 2D) and between the plasma levels of log10VEGF-A and urine log10(Alb/Cre; $r=-0.44$; $P=0.002$; Figure 2E). Thus, the circulating levels of sFlt-1 are significantly positively related to the degree of proteinuria, whereas the circulating levels of VEGF-A are significantly inversely related to both blood pressure and proteinuria. Our results indicate that decreased levels of circulating VEGF-A cause the increases in blood pressure seen in the mice administered Ad m-sFlt-1.

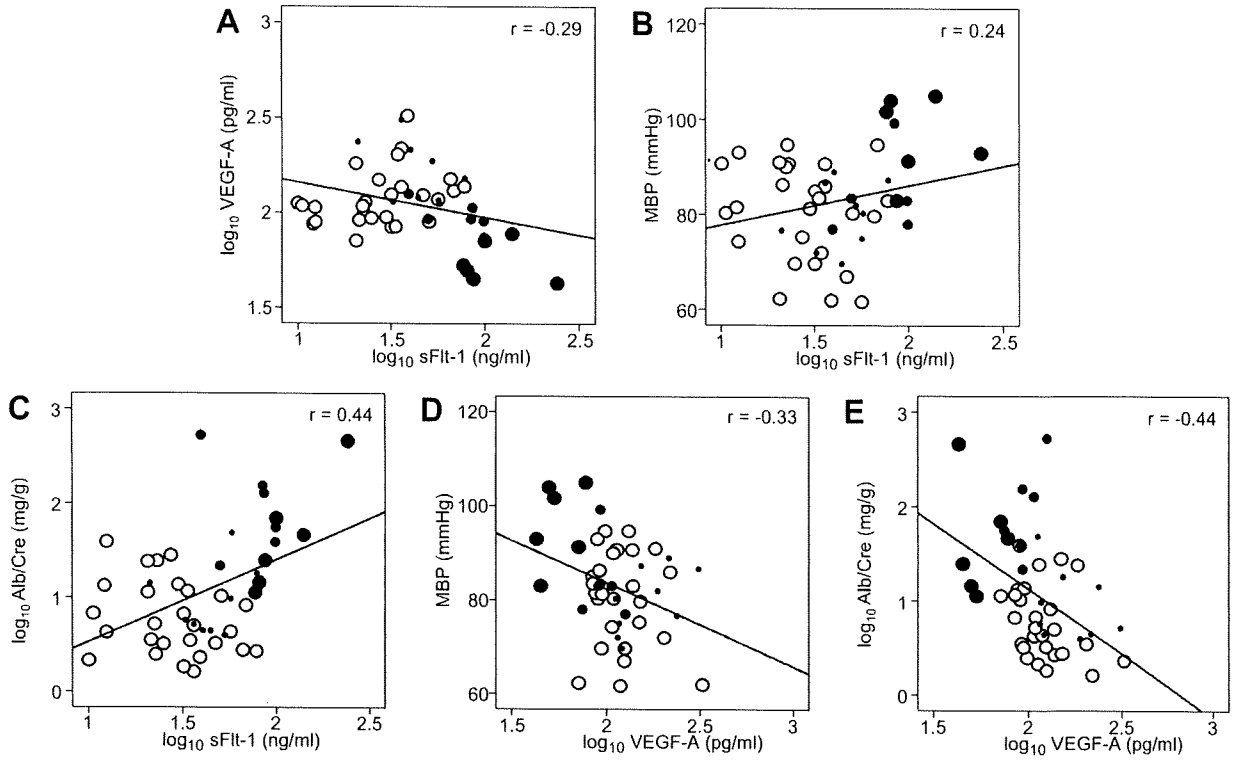

Figure 2. Scattergrams showing the relationship between the plasma levels of log10sFlt-1 and log10VEGF-A (A; $r=-0.29$; $P=0.042$), between the plasma levels of log10sFlt-1 and MBP (B; $r=0.24$; $P=0.098$), between the plasma levels of log10sFlt-1 and urine log10(Alb/Cre) (C; $r=0.44$; $P=0.002$), between the plasma levels of log10VEGF-A and MBP (D; $r=0.33$; $P=0.023$), and between the plasma levels of log10VEGF-A and urine log10(Alb/Cre) (E; $r=-0.44$; $P=0.002$). The large open circles represent combined data from the control mice and mice administered low, medium, and high doses of Ad LacZ. The small, medium, and large closed circles represent data from mice administered low, medium, and high doses of Ad m-sFlt-1, respectively.

Effects of rmVEGF164 and rmPlGF-2 in Mice Administered High-Dose Ad m-sFlt-1

In pregnant mice administered high-dose Ad m-sFlt-1 compared with mice administered vehicle, the levels of mouse VEGF-A (pg/ml) 3 to 4 hours after the last administration of rmVEGF164 were significantly increased (356 [121 to 793] versus 59 [56 to 68]; $P=0.010$; Figure 3A), and the level of mouse PlGF-2 (pg/ml) 3 to 4 hours after the last administration of rmPlGF-2 was also significantly increased (244 [133 to 244] versus 60 [52 to 74]; $P=0.010$; Figure 3B).

The rmVEGF164 treatment significantly decreased the MBP (mm Hg) on day 16.5 or 17.5 of gestation compared with the vehicle treatment (85±4 versus 97±2; $P=0.018$; Figure 3C). The rmPlGF-2 treatment also significantly decreased the MBP on day 16.5 or 17.5 of gestation compared with the vehicle treatment (86±3 versus 97±2; $P=0.018$). However, the urine Alb/Cre levels were not affected by treatment with either rmVEGF164 or rmPlGF-2 (Figure 3D). The rmVEGF164 treatment and rmPlGF-2 treatment did not ameliorate glomerular histology viewed by light microscopy in pregnant mice administered high-dose Ad m-sFlt-1 compared with those administered vehicle.

Discussion

In this study, we created a pregnant mouse model of preeclampsia, showing hypertension, proteinuria, and glomerular change, like endotheliosis, by transfecting a high dose (2×10^9 PFUs) of adenovirus encoding full-length m-sFlt-1. In addition, we revealed that rmVEGF164 and rmPlGF-2 ameliorate the hypertension induced by the administration of a high dose of Ad m-sFlt-1 in pregnant mice.

Development of a Pregnant Mouse Model of Preeclampsia Using Ad m-sFlt-1 and the Relationships Between the Serum Levels of m-sFlt-1/m-VEGF-A/m-PlGF-2 and Hypertension/Proteinuria

We, for the first time, created a mouse preeclampsia model using full-length m-sFlt-1 instead of truncated m-sFlt-1, which has been used in previous mouse preeclampsia models.[3,7,15–19] In the previous rat models using truncated m-sFlt-1, both hypertension and proteinuria emerged after low-dose (1×10^8-PFU) administration of Ad m-sFlt-1.[3] On the contrary, we needed a higher dose of Ad m-sFlt-1 to generate both hypertension and proteinuria. In our study, increases in the plasma levels of sFlt-1 were related to the occurrence of proteinuria, and decreases in the plasma levels of VEGF-A were related to the occurrence of both hypertension and proteinuria. Therefore, the circulating levels of sFlt-1 and VEGF-A may be important for the occurrence of hypertension and proteinuria. It is possible that the in vivo expression of sFlt-1 in the liver per administered dose of adenovirus or the circulating levels of sFlt-1 or VEGF-A were different between the 2 studies.

It is well known that the administration of sFlt-1 into rodents results in the occurrence of proteinuria.[3,7,16,25] Kamba

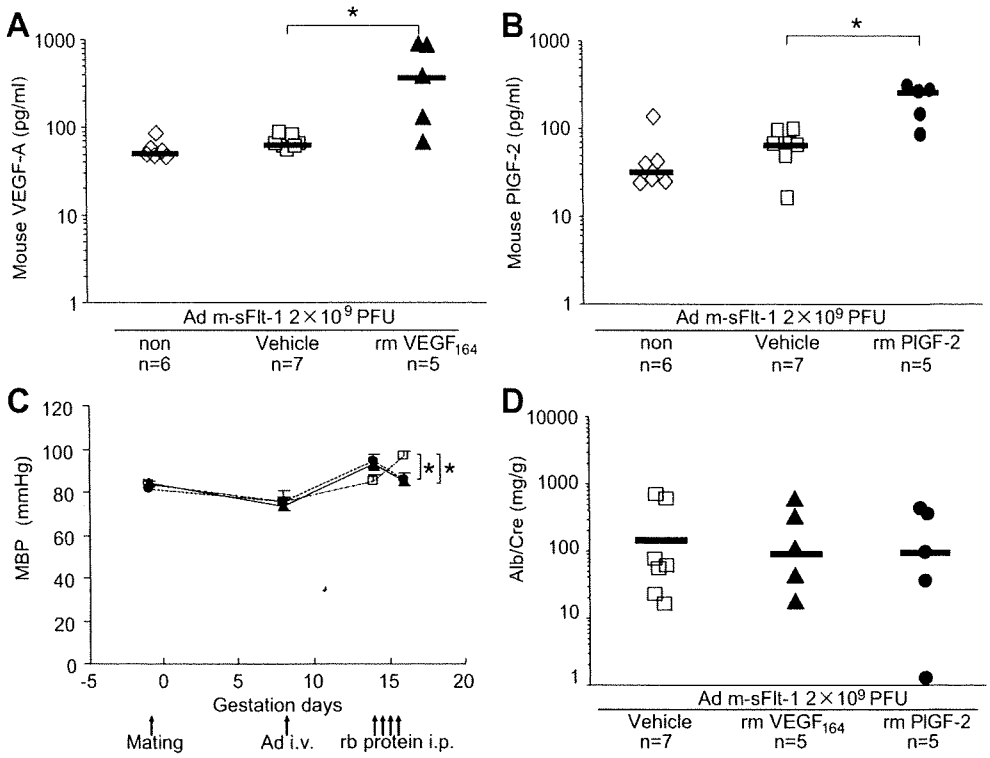

Figure 3. Data in pregnant mice administered high-dose Ad m-sFlt-1 after treatment with vehicle, rmVEGF164, or rmPlGF-2. A, The levels of mouse VEGF-A 3 to 4 hours after the last administration of nothing, vehicle (twice a day, 4 times), or rmVEGF164 (100 μg/kg, twice a day, 4 times). B, The levels of mouse PlGF-2 3 to 4 hours after the last administration of nothing, vehicle (twice a day, 4 times), or rmPlGF-2 (100 μg/kg, twice a day, 4 times). C, MBP (millimeters of mercury) during the prepregnancy period, just before the injection of Ad m-sFlt-1, just before the administration of recombinant proteins or vehicle, and at 1 to 2 hours after the last administration of recombinant proteins or vehicle. □, ▲, and ● represent the values in pregnant mice administered a high dose of Ad m-sFlt-1 with vehicle (n=7), rmVEGF164 (n=5), or rmPlGF-2 (n=5), respectively. D, Urine Alb/Cre ratios on day 16.5 or 17.5 of gestation in pregnant mice administered a high dose of Ad m-sFlt-1 with vehicle, rmVEGF164, or rmPlGF-2. *$P<0.05$.

et al[25] reported that proteinuria, but not hypertension, emerged in nonpregnant mice administered adenovirus-truncated m-sFlt-1. Sugimoto et al[7] reported that IV administration of an sFlt-1/Fc chimera protein into nonpregnant mice resulted in proteinuria 3 hours after the administration. Maynard et al[3] reported that IV administration of adenovirus-truncated m-sFlt-1 into nonpregnant and pregnant rats resulted in severe proteinuria. In addition, the effect of adenovirus-truncated m-sFlt-1 in pregnant rats on the occurrence of proteinuria was more severe than that of adenovirus-soluble endoglin, both of which can cause both hypertension and proteinuria in pregnant mice.[16] However, the proteinuria presented in this mouse model is fairly modest compared with what is presented in the rats where the Alb/Cre ratio is frequently >1000 mg/g.[3] One possibility is that the dose using the full-length sFlt1 is not enough, because it tends to have a poor bioavailability in contrast to truncated sFlt1. Another possibility is that proteinuria may depend on the background of the mouse strains used.

In our experiment, the decrease in the circulating levels of VEGF-A, but not PlGF-2, was related to the increase in MBP. The effect of VEGF-A on blood pressure has been reported in humans.[26–29] The blocking of signal transduction of VEGF-A by anti-VEGF monoclonal antibody[26,27] and tyrosine kinase inhibitors, such as soraferib[28] and sumitinib,[29] induces hypertension. On the contrary, the administration of rmVEGF-A results in a decrease of MBP in rats.[30,31] Thus, an appropriate circulating VEGF-A level appears to be important for the maintenance of normal blood pressure. Although the detailed mechanism by which a decreased level of VEGF-A affects the occurrence of hypertension has not been elucidated, the modulation of the production of NO by VEGF-A via endothelial NO synthase activity in endothelial cells may be related to the change in blood pressure.[32,33]

The mouse PlGF-2 was not altered in this mouse model of preeclampsia. The circulating levels of PlGF-1 are low in preeclamptic women,[3–5,19–21] because the human ELISA assay measured free PlGF but not total PlGF. We could not know whether the assay of the mouse PlGF-2 in our study measured a free or total PlGF-2 assay. We speculate that the assay of the mouse VEGF-A measured a free VEGF-A and that the assay of the mouse PlGF-2 measured a total PlGF-2, because the levels of VEGF-A were decreased but the levels of PlGF-2 were not decreased by the administration of high-dose Ad m-sFlt-1.

Effect of rmVEGF164 and rmPlGF-2 on Blood Pressure and Proteinuria in a Pregnant Mouse Preeclampsia Model

We revealed that rmVEGF164 ameliorates the hypertension induced by the administration of a high dose of Ad m-sFlt-1

in pregnant mice. Li et al[19] reported the therapeutic effect of VEGF-A in preeclamptic rat models induced by the IV administration of truncated Ad m-sFlt-1 on 8 days of gestation; the SC administration of 800 μg/kg per day of recombinant human VEGF121 for 6 days during day 11 to day 16 of gestation resulted in the amelioration of systolic blood pressure. In our study, the administered doses of rmVEGF164 were lower, and the treatment duration of rmVEGF164 was shorter compared with the study of Li et al[19]; however, the MBP after the administration of rmVEGF164 decreased. Therefore, the administration of recombinant VEGF-A to women with preeclampsia may be an effective treatment for this condition, especially for women with early onset preeclampsia, in whom the delay of birth by weeks may contribute to the reduction of neonatal complications and neonatal stay in the newborn intensive care unit.[34]

We, for the first time, revealed that rmPlGF-2 ameliorates the hypertension induced by high doses of Ad m-sFlt-1 in pregnant mice. To the best of our knowledge, this is the first experiment that showed the antihypertensive effect of rmPlGF on the hypertension induced by Ad m-sFlt-1 in pregnant mice. Hypotension induced by VEGF-A is mainly mediated by VEGFR2.[30] Because PlGF binds only to VEGFR1 and has little or no direct mitogenic or permeability-enhancing activity,[35,36] we supposed that the hypotensive effect of PlGF is very weak. However, surprisingly, the antihypertensive effect of PlGF was as strong as the antihypertensive effect of VEGF-A in our preeclampsia mouse model. Recently, Osol et al[37] reported that PlGF had a vasodilatory effect on numerous arteries and veins in rats; pregnancy significantly enhanced sensitivity to PlGF in rat uterine arteries; the vasodilatory effect of PlGF during pregnancy was mainly attributed to the activation of VEGFR1 but not VEGFR2; VEGFR1 was upregulated in the uterine artery wall during gestation; and PlGF dilation was principally mediated by the release of NO in rat uterine arteries. In addition, Osol et al[37] also showed that both rat mesenteric and human SC arteries dilated in response to PlGF in an NO-independent manner. These observations clearly suggest that PlGF has the ability to dilate vessels during pregnancy; that is, PlGF has a potentially antihypertensive effect during pregnancy.

Possible Mechanism by Which Hypertension and Proteinuria Emerge in a Pregnant Mouse Administered Recombinant sFlt-1

Recently, Bridges et al[8] reported that placental and vascular superoxide productions were increased and plasma VEGF-A concentrations were decreased in pregnant rats administered recombinant sFlt-1 chronically during days 13 to 18 of gestation. Vasorelaxations to both acetylcholine and sodium nitroprusside were decreased in pregnant rats administered recombinant sFlt-1, and the decrease of vasorelaxation to acetylcholine was attenuated by the addition of the superoxide scavenger Tiron, indicating elevated maternal sFlt-1, via the decrease of VEGF, results in increased oxidative stress that contributes to vascular dysfunction during pregnancy.[8] VEGF contributes to the maintenance of an appropriate balance of pro-oxidant and antioxidant factors via manganese superoxide dismutase[38] and NADPH oxidase,[39] and regulates NO production.[32,33] We also observed that the plasma VEGF levels were decreased in pregnant mice administered high doses of Ad m-sFlt-1 and the treatment of VEGF ameliorated hypertension induced by Ad m-sFlt-1. Therefore, increased oxidative stress and vascular dysfunction might be factors in hypertension in the present model, although we did not measure the oxidative stress. Taken together, VEGF antagonism may induce endothelial cell oxidative stress and contribute to renal dysfunction and hypertension.

Conclusions

We, for the first time, created a mouse preeclampsia model using full-length m-sFlt-1 instead of truncated m-sFlt-1, which has been used in previous mouse preeclampsia models.[3,7,15–19] Not only rmVEGF164, but also PlGF-2, ameliorated hypertension in the mouse preeclampsia model induce by full-length m-sFlt-1. Additional study of PlGF as a potential drug for preeclampsia is warranted.

Perspectives

Our study suggested a possible new therapy using PlGF for preeclamptic women. However, there are several unsolved issues. How many doses of PlGF-2 are sufficient for the amelioration of hypertension and proteinuria in the pregnant mouse model of preeclampsia using Ad m-sFlt-1? When should the administration of PlGF be started to restrict the occurrence of hypertension and proteinuria? What kinds of angiogenetic factors show the best therapeutic effects? In addition, if possible, we should make a mouse/rat model using adenoviral human sFlt-1, because the effect of m-sFlt-1 on mouse VEGF-A/PlGF might be different from that of human sFlt-1 on human VEGF-A/PlGF. If the human PlGF-1/PlGF-2 is used for the prevention/therapy of preeclampsia, we should carefully monitor the occurrence of possible adverse effects, such as lung edema, the development of new blood vessels in nontargeted tissues.

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Disclosures

None.

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