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Effects of KRN633, an Inhibitor of Vascular Endothelial Growth Factor Receptor-2 Tyrosine Kinase, on Vascular Development of Placenta and Fetus of Mid Pregnancy in Mice

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Abstract. Inhibition of the vascular endothelial growth factor (VEGF) signaling pathway during pregnancy contributes to several pathologic pregnancies, such as hypertension, preeclampsia, and intrauterine growth restriction, but its effects on the fetus have not been fully examined. To determine how inhibition of the VEGF signaling pathway affects the fetal vascular development of mid pregnancy, we treated pregnant mice daily with either the VEGF receptor-2 (VEGFR-2) tyrosine kinase inhibitor KRN633 (300 mg/kg, p.o.) or the vehicle from 13.5 to 15.5 day of pregnancy. On the 16.5 day of pregnancy, the vascular beds in the placenta and several organs of the fetus were visualized by fluorescent immunohistochemistry. All mice treated with KRN633 appeared healthy, and total numbers of fetuses per litter were unaffected. However, weights of the placenta and fetus from KRN633-treated mice were lower than those from the vehicle-treated ones. No external malformations and bleeding were observed in the placenta and fetus, whereas immunohistochemical analyses revealed that the vascular development in labyrinthine zone of placenta and fetal organs examined (skin, pancreas, kidney, and lung) were impaired by KRN633 treatment. These results suggest that inhibition of the VEGF signaling pathway during mid pregnancy suppresses vascular growth of both the placenta and fetus without obvious health impairments of mother mice and increases the risk of induction of intrauterine growth restriction.

Keywords: fetus, intrauterine growth restriction, placenta, vascular endothelial growth factor (VEGF), vascular development

Introduction

There is a growing body of evidence that perturbations of angiogenesis play an important role in the pathogenesis of various disorders such as cancer, retinopathies, rheumatoid arthritis, and endometriosis (1, 2). Several factors have been identified as important regulators of angiogenesis, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor, epidermal growth factor, and platelet-derived growth factor (2, 3). Blockade of the signaling evoked by these factors is an attractive strategy for treating these disorders (4). Among the factors known to stimulate the angiogenesis, the VEGF family may be the most extensively studied (5, 6). Recent investigations demonstrated that anti-VEGF therapy is considerably successful in treating patients with cancer (7) and wet age-related macular degeneration (8). Currently, small-molecule inhibitors of VEGF receptor (VEGFR) tyrosine kinase have developed as one promising approach to treat these diseases (9). On the other hand, inhibition of the VEGF signaling pathway frequently causes hypertension and proteinuria, possibly due to impairment of normal endothelial cell function (2, 10).

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In normal pregnancy, both VEGF and placental growth factor (PlGF) are thought to be important for embryonic development and placental growth (11, 12). The impaired function of these factors is associated with preeclampsia and intrauterine growth restriction (IUGR), both of which are serious problems of pregnancy. Accumulating evidence indicates that elevated serum level of soluble VEGFR-1 (soluble fms-like tyrosine kinase-1, sFlt-1) that binds free VEGF and PlGF is related to the pathogenesis of preeclampsia (10, 13 – 17). In pregnant animals, overexpression of sFlt-1 using adenoviral vectors induces proteinuria, hypertension, and glomerular endotheliosis, the hallmarks of preeclampsia, and the same procedure causes IUGR (10, 18).

It is well known that defective placental vascular development during pregnancy shows an embryonic lethal phenotype and IUGR (19, 20). Indeed, overexpression of sFlt-1 and administration of TNP-470, an angiogenesis inhibitor, in pregnant animals impaired the placental vascular development and caused IUGR (18, 21). Both decreased placental blood flow and the inhibitor delivered to the fetus may influence the fetal vascular development and result in changes to the architecture of the vascular network. However, there was no detailed examination of vasculature in the fetus in the previous studies.

The purpose of the present study, therefore, was to determine how inhibition of the VEGF signaling pathway affects the vascular development of the fetus. For this purpose, we used KRN633, a potent small-molecule inhibitor of VEGFR tyrosine kinase (22) and examined the effects of the inhibitor on the mid-pregnancy mice. Since the marked elevation of serum sFlt-1 in women with preeclampsia was frequently observed after the mid-pregnancy stage, we focused on the effects of VEGF inhibition on the fetus of mid pregnancy.

Material and Methods

Animals

Adult ICR mice were obtained from the Charles River Breeding Laboratories (Tokyo) and maintained on mouse chow (Oriental, Tokyo) and tap water ad libitum. Female animals (8 – 20 weeks of age) were placed with males overnight and vaginal plugs checked the following morning to determine if mating had occurred. The day of the appearance of the vaginal plug was considered as 0.5 day of pregnancy. All procedures involving animals were performed in accordance with the Guidelines for Animal Experiments in Kitasato University adopted by the Committee on the Care and Use of Laboratory Animals of Kitasato University.

Drugs and treatment

KRN633 (*N*-{2-chloro-4-[(6,7-dimethoxy-4-guinazolinyl) oxy]phenyl}-*N*′-propylurea), a potent small-molecule inhibitor of VEGFR-2 tyrosine kinase, was suspended in 0.5% methylcellulose in water and administered at a dose of 300 mg/kg body weight in a volume of 500 μl by gastric gavage once daily from day 13.5 to 15.5. In preliminary experiments, five different doses of KRN633 (10, 30, 100, 200, and 300 mg/kg) were tested. All doses of KRN633 induced IUGR; however, the most severe IUGR with a high-reproducibility was observed by treatment with the highest dose 300 mg/kg. Based on the results from the dose–response study, we chose the dose tested in this study. KRN633 was synthesized at Kitasato University.

Experimental procedures

Pregnant mice were treated with KRN633 (300 mg/kg in a volume of 500 μl) or its vehicle (0.5% methylcellulose, 500 μl) once daily from day 13.5 to 15.5. At day 16.5, mice were sacrificed to obtain placentas and fetuses. Fetuses were dissected in ice-cold PBS and skins, lungs, pancreases, and kidneys were collected. The collected fetal organs and placentas were fixed overnight in 4% paraformaldehyde in PBS. After fixation, skins were processed as whole mounts to visualize the three-dimensional structure of the microvasculature. Other organs of the fetuses and placentas were rinsed several times with PBS, infiltrated overnight with 30% sucrose in PBS at 4°C, and frozen in OCT compound (Sakura Finetek, Torrance, CA, USA).

Immunohistochemistry

Cryostat sections were cut with a cryostat and dried on glass slides. Thicknesses of section for placenta, lung and kidney, and pancreas were 20, 10, and 30 μm, respectively. Sections were rinsed of OCT compound and then incubated in blocking solution (5% normal goat serum or 5% normal hamster serum) in PBS containing 0.3% Triton X-100 (PBS / 0.3% Triton X-100) for 1 h at room temperature. To label endothelial cells, sections of fetal organs and placenta were incubated with a rat monoclonal anti-CD31 antibody (1:500, clone MEC 13.3; BD Pharmingen, San Jose, CA, USA) or hamster monoclonal anti-mouse CD31 antibody (clone 2H8; Chemicon, Temecula, CA, USA) overnight. After several rinses with PBS / 0.3% Triton X-100, sections were further incubated for 5 h with a FITC- or Cy3-labeled, species-specific secondary antibody against the CD31 antibody (anti-rat or anti-hamster) (1:400; Jackson ImmunoResearch, West Grove, PA, USA). Sections were rinsed in PBS / 0.3% Triton X-100 and mounted with Vectashield (Vector Laboratories, Burlingame, CA,

USA). Control sections incubated in the absence of primary antibodies were also processed and evaluated for specificity or background levels of staining.

The skin was incubated in blocking solution (5% normal goat serum) in PBS / 0.3% Triton X-100 for 1 h at room temperature. To visualize the vascular network, the skins were incubated with a rat monoclonal anti-CD31 antibody (1:500, BD Pharmingen) overnight, followed by incubation with a Cy3-conjugated goat antibody against rat immunoglobulins (1:400, Jackson ImmunoResearch). Tissues were rinsed in PBS / 0.3% Triton X-100 and the whole mounts were prepared with Vectashield (Vector Laboratories). In some experiments, sections of kidney or skins were incubated in a combination of rabbit polyclonal anti-type IV collagen antibody (1:8000; Cosmo Bio Co., Tokyo) and rat monoclonal anti-CD31 antibody (1:500) overnight. As described above, after several rinses with PBS / 0.3% Triton X-100, tissues were further incubated for 4 h with FITC- or Cy3-labeled, species-specific secondary antibodies against anti-type IV collagen antibody (anti-rabbit) and anti-CD31 antibody (anti-rat). In negative controls, the primary antibodies were replaced in PBS and background staining levels were evaluated.

Images were taken by using a fluorescent microscope system, BZ-9000 (Keyence, Osaka) or a confocal laser scanning microscope, LSM 510 Meta (Zeiss, Oberkochen, Germany).

Quantitative assessment of fetal vascular development

Images that were obtained from each section or skin whole-mount were analyzed using ImageJ (http://rsb.info.nih.gov/ij/). Based on fluorescence intensities ranging from 0 to 255, blood vessels were distinguished from background by empirically determining threshold values that included only blood vessels in specimens. The area density of blood vessels stained with anti-CD31 antibody was calculated as the proportion of pixels having a fluorescence intensity value equal to or greater than the corresponding threshold. For assessment of renal glomerular capillaries, the CD31-immunoreactive vascular area in Bowman's capsules was evaluated (10 – 30 Bowman's capsules per fetus). Mean values were calculated for organs in each fetus (8 fetuses in each group) and from these values overall means were calculated for each group (4 mother mice per group).

Measurement of cross-sectional area of placenta

Images were obtained from two separate placental sections that represent middle regions of each placenta. Using the ImageJ software, the total cross-sectional area of the placenta and cross-sectional areas of decidual and labyrinthine zones were measured. Mean values were calculated for each placenta (23 – 28 placentas in each group) and from these values overall means were calculated for each group (7 mother mice per group).

Assessment of kidney function and measurement of glucose and hemoglobin in blood

Urine and blood samples were obtained at the same time as the placentas and fetuses were taken. Because proteinuria is frequently observed in mice after inhibition of the VEGF signaling pathway by overexpression of sFlt-1, anti-VEGF antibody, and VEGFR tyrosine kinase inhibitors (23, 24), urine was tested for protein using test strips (Multistix SG-L; Bayer Medical, Ltd., Tokyo). Inhibition of the VEGF signaling pathway for 1 – 3 weeks induces capillary regression in pancreatic islets and affects glucose metabolism (24). Therefore, the concentration of blood glucose was measured using a glucometer (Ascensia BREEZE, Bayer Medical Ltd.). We also measured the hemoglobin concentration in the blood obtained from fetuses and the mother mice by the cyanomethemoglobin method (Hemoglobin Test Wako; Wako Fine Chem., Tokyo).

Statistical analyses

Statistical comparison of paired data was performed with Student's *t*-test, and multigroup data were analyzed by ANOVA (one-way) followed by the Bonferroni's post-test using PRISM4 software (GraphPad Software, Inc., San Diego, CA, USA). A P value smaller than 0.05 was considered to be statistically significant. All values are presented as the mean ± S.E.M.

Results

All pregnant mice treated with KRN633 appeared healthy and there were no significant differences in body weight and concentrations of glucose and hemoglobin in blood between vehicle- and KRN633-treated mice (body weight at day 16.5: vehicle, 53 ± 2 g, n = 7 vs. KRN633, 49 ± 2 g, n = 7; glucose: vehicle, 99.5 ± 6.2 mg/dl, n = 4 vs. KRN633, 95.8 ± 4.5 mg/dl, n = 4; hemoglobin: vehicle, 18.3 ± 1.0 mg/dl, n = 4 vs. KRN633, 18.0 ± 0.5 mg/dl, n = 4). The levels of protein in the urine of KRN633-treated mice were similar to those in mice treated with vehicle (data not shown). Total numbers of fetuses per litter were not affected significantly (number of fetuses: vehicle, 14 ± 1 vs. KRN633, 15 ± 1). No external malformations and bleeding were observed in the fetuses and placentas of pregnant mice treated with KRN633 macroscopically; however, the fetuses of the treated group looked smaller than those of the control group (Fig. 1A). As summarized in Fig. 1B, the weight of fetuses that were obtained from KRN633-treated mice

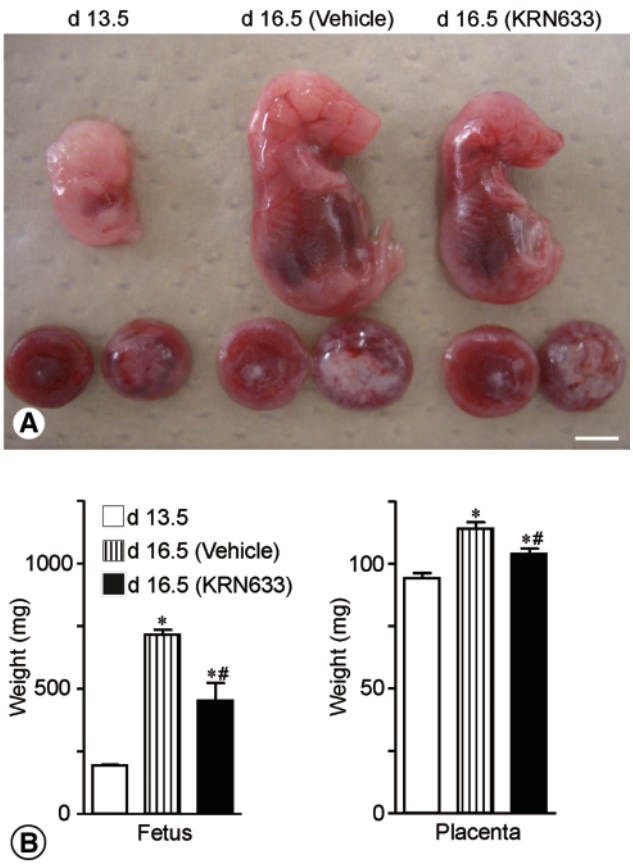

Fig. 1. Effects of KRN633 on fetuses and placentas of pregnant mice. Pregnant mice were treated with KRN633 (300 mg/kg, p.o.) or the vehicle from 13.5 to 15.5 day of pregnancy and mice were sacrificed at 16.5 day. For comparison, seven pregnant mice were sacrificed at 13.5 day of pregnancy. No external malformations and bleeding were observed in the fetuses and placentas of pregnant mice treated with KRN633 macroscopically, but the fetuses of the KRN633-treated group were smaller than those of the vehicle-treated group (A). Significant increases in the weights of the placenta and fetus from both vehicle- and KRN633-treated animals were observed compared to 13.5 day of pregnancy (B). The weights of the placenta and fetus of KRN633-treated pregnant mice were lower than those of vehicle-treated mice (B). Each column with a vertical bar represents the mean ± S.E.M. from fetuses (number of fetuses: day 13.5, 90; vehicle, 87; KRN633, 102) or placentas (number of placentas: day 13.5, 62; vehicle, 58; KRN633, 87) obtained from seven mothers in each experimental group. $*P < 0.05$ vs. day 13.5, $^{\#}P < 0.05$ vs. day 16.5 (Vehicle). Bar, 500 μm (A).

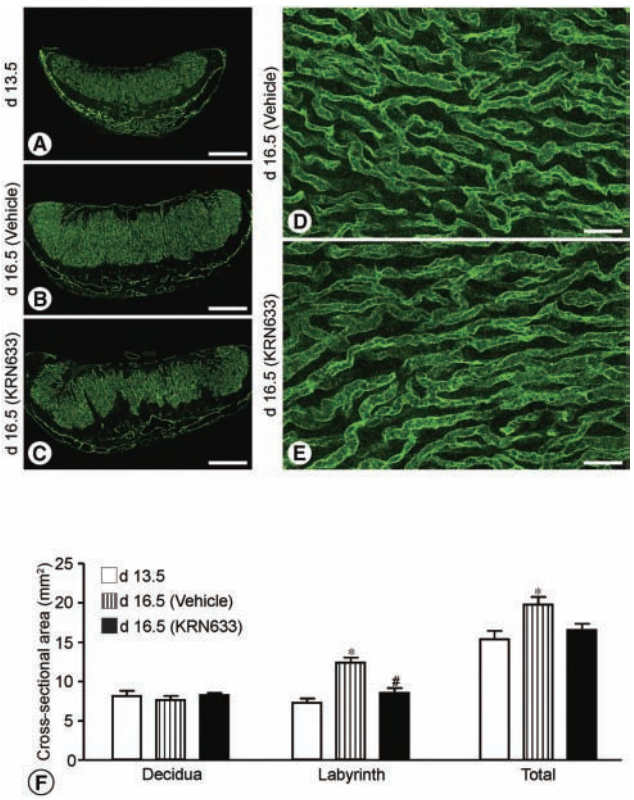

Fig. 2. Overview of placental vasculature labeled with anti-CD31 antibody at 13.5 and 16.5 day (with/without KRN633 treatment) of pregnancy. Treatment with KRN633 markedly compromised the development of labyrinthine vascular bed (A – C). No morphological differences in blood vessels of the labyrinthine layer between vehicle- and KRN633-treated groups were detected (D and E). The increase in cross-sectional area of the placenta (Total) and the labyrinthine zone (Labyrinth) was attenuated by KRN633 treatment (F). There was no significant difference in cross-sectional area of the decidual zone (Decidua) among the groups. Each column with a vertical bar represents the mean ± S.E.M. $*P < 0.05$ vs. day 13.5, $^{\#}P < 0.05$ vs. day 16.5 (Vehicle). Bar, 1.3 mm (A – C); 51 μm (D and E).

was significantly lower than those from vehicle-treated ones, although their body weights were greater than ones of fetuses at day 13.5. Similarly, placentas from both vehicle- and KRN633-treated animals were larger than those from 13.5 day of pregnancy. The weights of placentas of KRN633-treated pregnant mice were significantly lower than those of vehicle-treated pregnant mice (Fig. 1B). An increase in blood hemoglobin concentration, which is recognized as a compensatory response to chronic tissue hypoxia, was found in fetuses from KRN633-treated mice (fetuses in vehicle-treated group, 9.9 ± 0.4 mg/dl, n = 14 vs. fetuses in KRN633-treated group, 11.6 ± 0.9 mg/dl, n = 14, $P < 0.05$).

The cross-sectional images of placenta showed that KRN633 treatment did not largely affect the overall size of placenta, whereas it compromised the development of labyrinthine vascular bed (Fig. 2: A – C). Morphological changes in blood vessels of the labyrinthine layer between vehicle- and KRN633-treated groups were not detected by immunohistochemical techniques (Fig. 2: D and E). KRN633 significantly attenuated the increase in cross-sectional area of the labyrinthine zone, whereas no significant difference in total cross-sectional area of the placenta was observed between vehicle- and KRN633-treated mice (Fig. 2F). There was no significant differ-

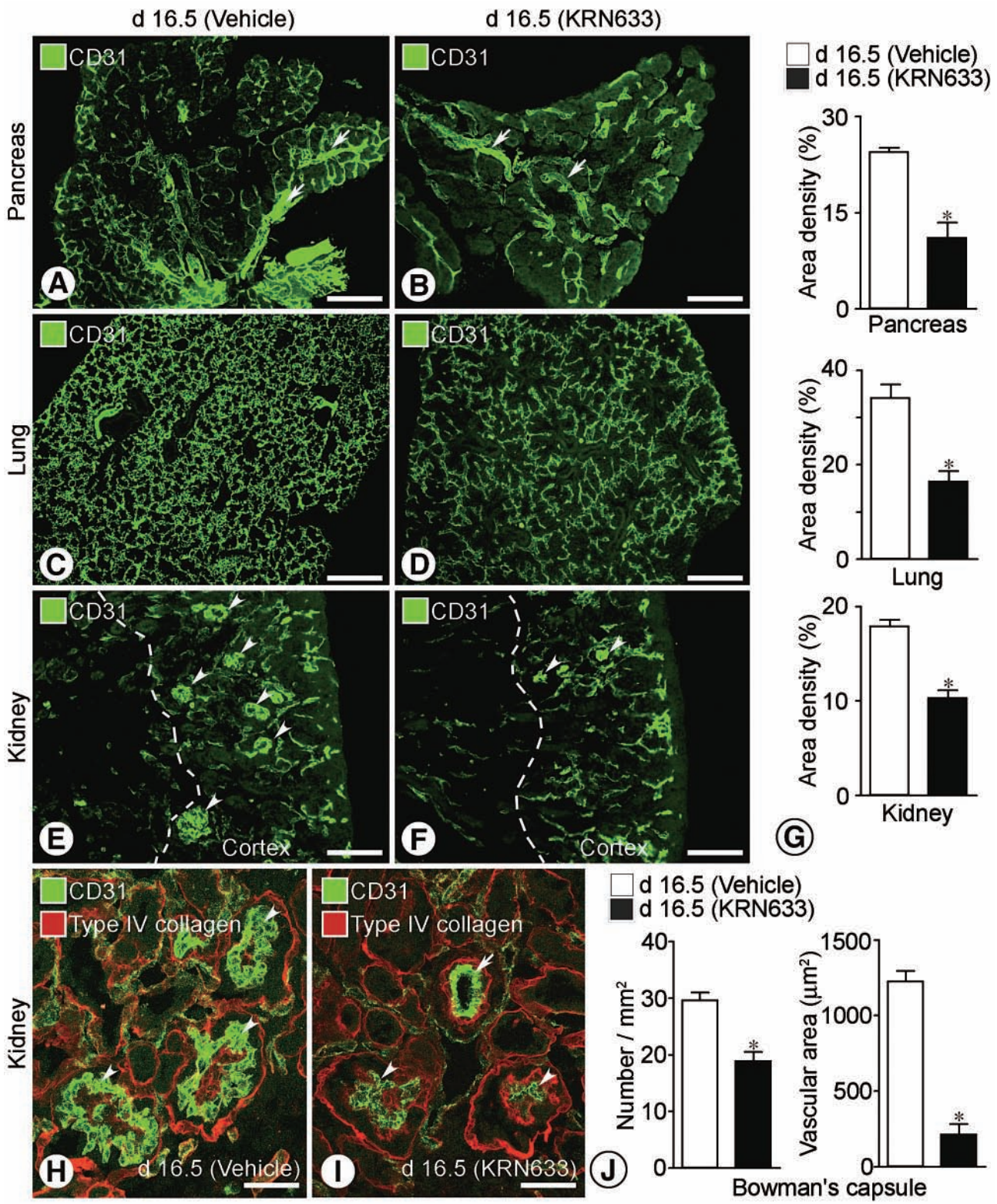

Fig. 3. Decrease in vascularity in fetal organs from pregnant mice treated with KRN633. Fluorescence micrographs show the vasculature labeled with anti-CD31 antibody in the pancreas, lung, and kidney of fetus from vehicle- and KRN633-treated mice (A – F). VEGF inhibition resulted in pruning of the capillary network in pancreas, lung, and kidney and reduced the vascularity of these organs (B, D, F, and G). Large vessels (arrows) were resistant to the VEGF inhibitor (B and I). The number of glomerular capsules and renal glomerular capillaries (arrow heads) were decreased (E, F, and H – J). Each column with a vertical bar represents the mean ± S.E.M. *$P < 0.05$ vs. day 16.5 (Vehicle). Bar, 220 μm (A – D); 110 μm (E and F); 72 μm (H and I).

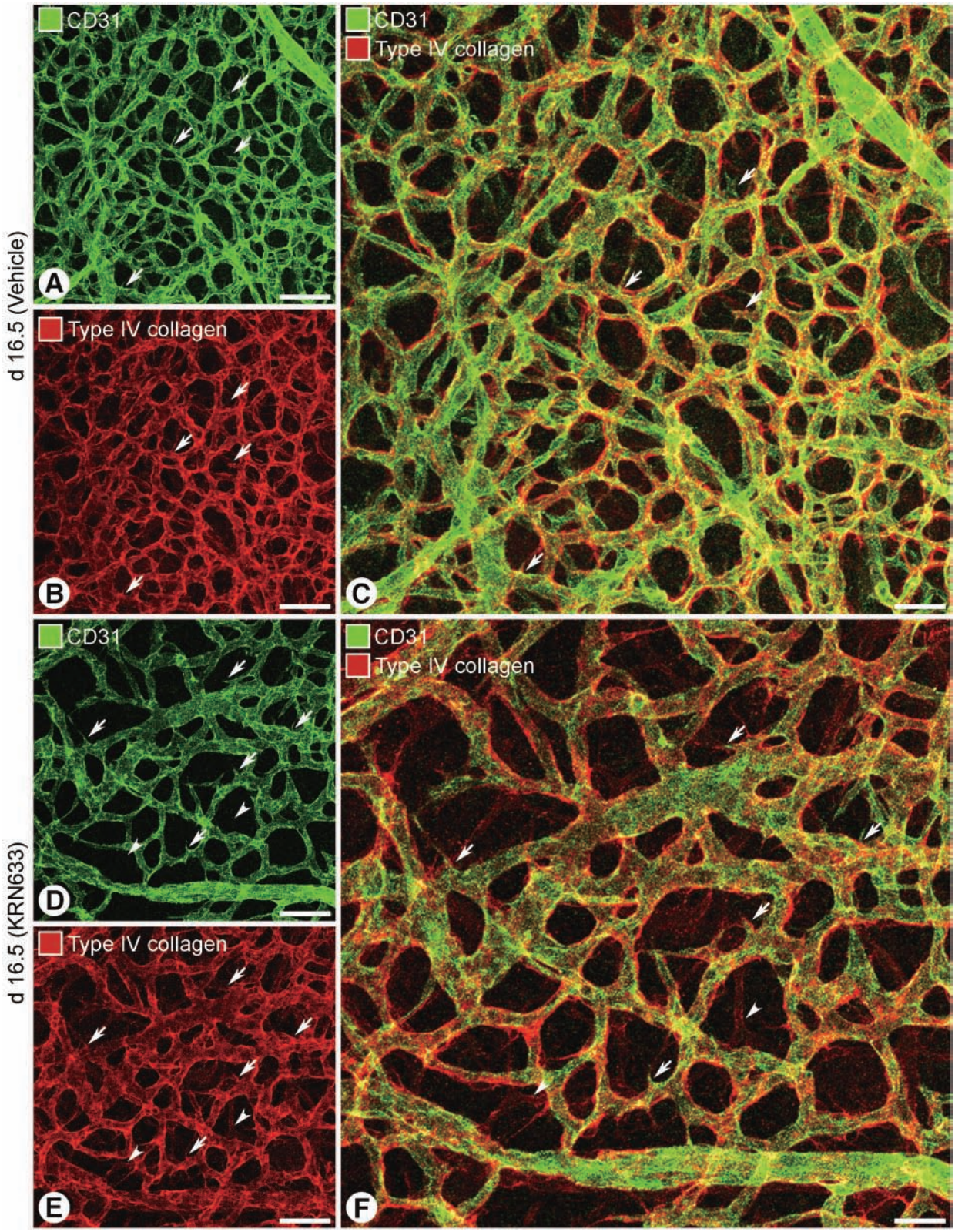

Fig. 4. Fetal microvasculature in whole-mounts of ear skin. The density of blood vessels stained with antibodies to CD31 was reduced in the fetus from KRN633-treated mice (A and D). We observed that the endothelial cells at the tips of vascular sprouts extended long filopodia in both fetuses from vehicle- and KRN633-treated mice (A – F, arrows). Image showing fetal ear skin vasculature stained for CD31 and type IV collagen immunoreactivities indicates the presence of empty sleeves of basement membrane (arrowheads) after KRN633 treatment (D – F). Bar, 110 μm (A, B, D, and E); 38 μm (C and F).

ence in cross-sectional area of the decidual zone among the groups.

To address the question of how VEGF inhibition affects the microvasculature in fetus of mid-pregnancy mice, we evaluated the CD31-immunoreactive blood vessels in fetal pancreas, lung, and kidney. At day 16.5, the dense capillary network was found in these organs of normal fetus but in the treated mice, the vascular densities were significantly reduced (Fig. 3: A – G). The number of glomerular capsules and renal glomerular capillaries labeled with anti-CD31 antibody were decreased markedly (Fig. 3: E, F, and H – J).

Using whole-mounts of ear skin stained with antibody to CD31, the three-dimensional structure of fetal microvasculature was examined in more detail. A dense network of capillaries was found at day 16.5 (Fig. 4A). In the skin vasculature of fetus from KRN633-treated mice, the normally dense capillary networks were simplified and the density was reduced (Vehicle, 82.5 ± 2.9% vs. KRN633, 48.3 ± 4.7%, n = 6 fetuses) (Fig. 4D). High-resolution analysis revealed that the endothelial cells at the tips of vascular sprouts extended long filopodia in both fetuses from vehicle- and KRN633-treated mice (Fig. 4: A – F). In the treated mice, sleeves of vascular basement membrane, identified by type IV collagen immunoreactivity, which persisted after blood vessels had regressed, serving as a historical record of the original blood vessel location, were observed (Fig. 4: D – F).

Discussion

The present study demonstrates that treatment with the small-molecule VEGFR-2 tyrosine kinase inhibitor KRN633 of mid-pregnancy mice induces IUGR. The immunohistochemical analysis indicates that the growth of blood vessels labeled with anti-CD31 antibody is greatly prevented in several organs of the fetus, as well as the labyrinthine zone of the placenta. These results suggest that inhibition of the VEGF signaling pathway at this stage of pregnancy reduces the blood supply to fetal tissues due to the diminished vascularization in both placenta and fetal organs and consequently increases the risk of induction of IUGR.

A previous study has shown that administration of the angiogenesis inhibitor TNP-470 to pregnant mice from day 10.5 to 18.5 results in diminished placental and fetal growth (21). The morphological analysis performed by the authors revealed that TNP-470 disturbed labyrinthine blood vessel growth in the placenta. Similarly, studies on pregnant animals injected with an adenoviral vector encoding sFlt-1 demonstrated that sizes of both placentas and fetuses were smaller in the treated mice when compared with the control (18). In the present study, by injecting the VEGFR-2 tyrosine kinase inhibitor KRN633 to pregnant mice, we also obtained similar results in relation to lower placental and fetal weights. In addition to these results, clinical findings also indicate that the impairment of placental development could increase the risk of preeclampsia and IUGR (25 – 28).

However, the question of how the formation of vasculature is affected in the growth-restricted fetuses remains to be fully elucidated. The gene knockout studies have been shown the critical role of VEGF in vascular development of embryos at the early stage of pregnancy (29 – 32). Even at the stage of mid-pregnancy, expression of VEGF was detected in fetal organs, including the lung, pancreas, and kidney (33 – 35). Therefore, inhibition of the VEGF signaling pathway in the fetus of mid pregnancy might impair the vascular development. The present study clearly demonstrates that the vascular density was reduced in organs of fetuses obtained from KRN633-treated mice; in particular, capillaries were more vulnerable to the treatment than larger vessels. Furthermore, the sleeves of vascular basement membrane, serving as a historical record of the original blood vessel location (36), were observed in the fetal ear skin vasculature stained with antibodies for CD31 and type IV collagen.

The impairment of the vascular network observed in the fetal organs appears to be attributed to two mechanisms, that is, suppression of new vessel formation and regression of preexisting blood vessels, but the observations resemble those in previous studies using multiple approaches that block the VEGF signaling pathway (24, 37). Therefore, it is likely that KRN633 exerts the effects on the fetus at least in part through blockade of the VEGF signaling pathway, although the secondary effect caused by failure of normal placental development cannot be ruled out. The high-magnification images revealed the existence of filopodial protrusions from endothelial cells in skin vasculature of the treated mice. This may imply that inhibition of VEGFR tyrosine kinase under our experimental conditions was insufficient to completely prevent the angiogenesis in fetal organs.

Because the expression level of VEGF in the labyrinthine zone is much higher than those of spongiotrophoblast and decidual zones (38), the vasculature in the labyrinthine zone would be susceptible to anti-VEGF agents. The results that the development of labyrinthine vascular bed was largely compromised in KRN633-treated mice support this hypothesis.

KRN633 is highly selective for VEGFR-2 tyrosine kinase; however, it also could inhibit other tyrosine kinases (VEGFR-1, VEGFR-3, PDGFR-β, etc.) (22). Therefore, some effects of KRN633 observed in our present in vivo study may be mediated partly through

inhibition of other tyrosine kinases. However, we found that even low doses of KRN633 (10 – 100 mg/kg per day), which could prevent tumor angiogenesis (22), significantly caused the restriction of fetal growth. For example, when 10 mg/kg per day of KRN633 was administered to pregnant mice from day 13.5 to the completion of delivery, approx. 10% reduction in body weight of the neonates was observed. Therefore, inhibition of VEGFR-2 tyrosine kinase might be partly responsible for the impairment of vascular network formation observed in this study.

Based on clinical findings, concentrations of sFlt-1 in serum from women with preeclampsia were higher than normal controls at the stages of mid- and late-pregnancy (2, 14 – 16). The risk of IUGR is higher in pregnant women with preeclampsia (39). In pregnant rats and mice injected with vector encoding the sFlt-1, the marked elevation of serum sFlt-1 at day 16 – 17 of pregnancy, the hallmarks of preeclampsia, such as hypertension and proteinuria, and restriction of fetal growth were observed (10, 18). Therefore, the diminished VEGF actions after mid-pregnancy appear to be associated with the pathogenesis of preeclampsia and the increase in risk of IUGR. In the present study, despite the marked impairment of vascular networks in placenta and fetal organs, KRN633-treated mice had a normal vascular network in the pancreas (unpublished observations) and no obvious health impairment, such as loss of body weight and impairment of renal function, possibly due to the short-term treatment. This suggests that the blood vessels in placenta and fetal organs could be markedly impaired by VEGF inhibitors at concentrations much lower than those needed to impact the preexisting blood vessels in mother mice. Accordingly, pregnant women with a mild or no significant increase in serum sFlt-1 levels and without signs of preeclampsia may also have the risk of induction of IUGR through an impairment of vascularization in both placenta and fetal organs.

IUGR contributes to a predisposition for hypertension, diabetes, coronary heart disease, and stroke during adulthood (40, 41). The endothelial dysfunction plays an important role in the onset and progression of these diseases. Our findings may imply that an incomplete vascular network formation in the pancreas and kidney at the fetal stage due to reduction in VEGF signaling contributes to increase the risk of appearance of several diseases during adulthood.

In conclusion, we found that treatment of mid-pregnancy mice with the small-molecule VEGFR-2 tyrosine kinase inhibitor KRN633 impairs vascularization in both placentas and fetal organs with no obvious health impairment and increase the risk of induction of IUGR.

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