Treatment of Newborn Mice with Inhibitors of Vascular Endothelial Growth Factor Receptor Tyrosine Kinase Induces Abnormal Retinal Vascular Patterning

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We have previously reported that treatment of newborn mice with KRN633, a vascular endothelial growth factor (VEGF) receptor tyrosine kinase inhibitor, delayed retinal vascularization leading to abnormal retinal vascular growth and patterns. To determine whether similar abnormalities are observed in newborn mice treated with other VEGF receptor tyrosine kinase inhibitors, we administered axitinib to mice on the day of birth and on the following day. When compared with control pups, a significant delay in retinal vascularization was observed in pups treated with axitinib (5 mg/kg). Axitinib-treated pups had a very dense capillary network on postnatal day (P) 6 and fewer central arteries and veins on P8 and P12. Central veins, but not arteries, were significantly enlarged on P8. These abnormalities were similar to those observed in KRN633-treated pups and probably represent a common phenotype induced by short-term treatment with VEGF receptor inhibitors in newborn mice. Therefore, mice treated postnatally with VEGFR inhibitors could serve as an animal model for studying the mechanisms of retinal vascular formation and patterning.

Key words axitinib; vascular development; vascular patterning; vascular endothelial growth factor; retina

The retinal vascular system is established during the first three weeks of life in mice. During the first week, the superficial vascular plexus forms and spreads radially from the optic nerve head towards the periphery of the ganglion cell layer. Over the following two weeks, the superficial vessels branch, growing perpendicularly to the primary vascular network, and penetrate the inner retinal layers along the outer edge of the inner nuclear layer.1–3

Vascular endothelial growth factor (VEGF) and its receptor (VEGFR) play a critical role in retinal vascular formation.1,4 In a recent study, we have shown that short-term interruption of retinal vascular development in newborn mice by injecting KRN633 (N-[2-chloro-4-[(6,7-dimethoxy-4-quinazolinyl)-oxy]phenyl]-N’-propylurea), an inhibitor of VEGF tyrosine kinase,5,6 induces abnormal retinal vascular growth and patterns.6,9 The KRN633-treated pups did not exhibit neovascular tufts on the vitreal surface of the retina, a characteristic finding in the oxygen-induced retinopathy (OIR) mouse model for proliferative ischemic retinopathy.5,7,8 However, they shared several features with the OIR model, in addition to exhibiting additional phenotypic features such as decreased numbers of central arteries and veins and enlargement of central veins.6 A detailed analysis of retinal vascular formation after short-term administration of KRN633 would be useful in clarifying the mechanisms underlying abnormal vascular formation and patterning in the retina. However, it is not known whether the vascular abnormalities found in KRN633-treated mice also occur in pups treated with other VEGFR inhibitors.

The purpose of the present study was to determine whether other VEGFR inhibitors produce similar abnormalities in retinal vascular patterns to those observed in KRN633-treated pups. For this purpose, newborn mice were treated with axitinib, a VEGFR inhibitor, and their retinal vascular growth and patterns were examined. Axitinib is a potent and selective inhibitor of VEGFR tyrosine kinases and has been shown to inhibit cellular autophosphorylation of VEGFR-1, -2, and -3 at picomolar concentrations.9,10

MATERIALS AND METHODS

Animals All animal procedures were performed in accordance with the Association for Research in Vision and Ophthalmology Statement on the Use of Animals in Ophthalmic and Vision Research, and the Regulations for the Care and Use of Laboratory Animals in Kitasato University used by the Institutional Animal Care and Use Committee for Kitasato University.

Adult ICR (Institute of Cancer Research) mice were obtained from the Charles River Breeding Laboratories (Tokyo, Japan) and maintained on a standard diet (Oriental Yeast, Tokyo, Japan) and tap water ad libitum. Female animals (age, 8–20 weeks) were placed with males, and pregnant females were then removed and placed in separate cages. Daily inspections were performed to determine the day of birth. The day of birth was defined as postnatal day (P) 0.

Effects of Axitinib on the Development of the Retinal Vasculature Newborn mice were treated with axitinib (5 mg/kg) on P0 and P1. Axitinib (Sigma-Aldrich, St. Louis, MO, U.S.A.) was dissolved in dimethyl sulfoxide (10 mg/mL), diluted in 4% EtOH/5% Tween 80/5% polyethylene glycol (PEG) 400, and administered subcutaneously at a dose of 10 µL/g body weight. Control animals were injected with the vehicle alone, but were treated identically to the axitinib-treated animals in all other respects. The dose of axitinib was selected based on our preliminary studies showing that 5 mg/kg was a maximum effective dose for our purpose.

Animals were sacrificed by using an overdose of sodium pentobarbital (Nacalai Tesque, Kyoto, Japan) on P2 (vehicle,
Detailed assessments were performed on P8 and P12; these days were selected because in axitinib-treated mice, P8 was the first day on which central arteries and veins could be clearly identified and P12 represented the point at which the retinal vasculature had reached the peripheral edge of the retina. The eyes were enucleated and fixed for 0.5–1 h at 4°C in 4% paraformaldehyde in phosphate-buffered saline (PBS). After fixation, the cornea, lens, uvea, and sclera were removed. The remaining retinas were post-fixed and stored in methanol at −20°C.

**Quantitative Analysis of the Retinal Vasculature** Immunohistochemical staining and quantitative analyses of the retinal vasculature were performed as previously described. In brief, retinas were incubated in blocking solution (5% normal goat serum) diluted in PBS containing 0.5% Triton X-100 (Nacalai Tesque) (PBS-T) for 0.5–1 h at room temperature. The retinas were incubated overnight at room temperature with a rat monoclonal anti-CD31 antibody (1:500, clone MEC 13.3; BD Pharmingen, San Diego, CA, U.S.A.) diluted with 5% normal serum in PBS-T. After incubation with the primary antibody, retinas were rinsed with PBS-T and further incubated for 4 h at room temperature with a Cy3-conjugated goat antibody raised against rat immunoglobulins (1:400; Jackson ImmunoResearch, West Grove, PA, U.S.A.) diluted in PBS-T. Tissues were rinsed in PBS-T and flat-mounts were prepared using Vectashield mounting media (Vector Laboratories, Burlingame, CA, U.S.A.). Images were obtained using a fluorescent microscope system (BZ-9000; Keyence, Osaka, Japan).

The areas of the retina covered by the developing vascular bed were identified using ImageJ software (http://rsb.info.nih.gov/ij/), and the size of this area relative to that of the retina (retinal surface area) was determined as a measure of retinal vascularization. The area densities of blood vessels stained with anti-CD31 antibody were measured using ImageJ. The total numbers of central arteries and veins were counted, and their diameters were measured.

**Statistical Analysis** Statistical comparisons of paired data were performed using Student’s t-test (GraphPad, San Diego, CA, U.S.A.). A p value less than 0.05 was considered to be statistically significant. All values are presented as the mean±S.E.
RESULTS

In vehicle-treated pups, retinal blood vessels grew outwards from the optic nerve towards the peripheral retina and had nearly reached the retinal edge by P8 (Figs. 1Aa–f, B). In pups treated with axitinib (5 mg/kg), fewer growing blood vessels had been observed around the optic nerve head on P2 (Fig. 1Ah) and network formation was markedly delayed (Figs. 1Ah–m, B). Despite the delayed vascularization, vascular density rapidly increased and was significantly higher in 6-d-old axitinib-treated pups when compared to age-matched vehicle-treated pups (Figs. 1C, D). By P4, a capillary-free space had become evident around the optic nerve head and along the central arteries in vehicle-treated pups, allowing central arteries and veins to be morphologically distinguished (Fig. 1Ab). In contrast, in axitinib-treated pups, remodeling of the capillary plexus was delayed, and no capillary-free space was observed on P6 (Fig. 1Aj); central arteries and veins could not be distinguished until P8 (Figs. 1Ak–m).

Analysis revealed that the axitinib-treated pups had fewer arteries and veins than vehicle-treated pups at both P8 and P12 (Figs. 2A, B). Central veins, but not arteries, of axitinib-treated pups were significantly enlarged on P8, whereas vein enlargement was no longer observed by P12 (Figs. 2A, C).

DISCUSSION

The present study demonstrates that newborn mice treated with axitinib, a VEGFR tyrosine kinase inhibitor, (1) delayed vascularization, (2) increased capillary density, (3) fewer central arteries and veins, and (4) enlargement of the central veins. These findings are consistent with abnormalities observed in mice treated with KRN633, another VEGFR inhibitor.6) Thus, a disturbance in normal vascular development in the newborn mouse as a result of short-term treatment with VEGFR inhibitors appears to induce abnormal vascular patterns in the retina. Mice treated postnatally with VEGFR inhibitors share several features with the OIR mouse model in which neovascularization is caused by retinal ischemia, but exhibit some differences in phenotype, including fewer central arteries and veins, and enlargement of central veins. These mice could serve as an animal model for studying the mechanisms involved in retinal vascular formation and patterning. They may also be useful for evaluating the efficacy of antiangiogenic compounds.

Axitinib-treated pups had a very dense capillary network on P6, but the dense vasculature normalized as the area of the retina covered by the vascular bed increased. This could suggest that the increase in vascular density is correlated with the degree of retinal vascular growth delay. Vascular insufficiency causes tissue hypoxia, which leads to an elevation in VEGF expression, stimulating endothelial cell proliferation.1) Therefore, it is possible that the delayed retinal vascularization found in axitinib-treated mice contributes to the induction of retinal hypoxia, leading to elevated VEGF expression and increased vascular density. The coverage of the entire retinal surface area by the vascular bed would result in recovery from retinal hypoxia, with a concomitant decrease in VEGF expression, leading to normal vascular density.

Another interesting phenotypic feature in pups treated with VEGF inhibitors was the enlargement of central veins on P8. The underlying mechanism remains unclear, but the impairment of pericyte recruitment may have contributed to central vein enlargement; disturbances in the recruitment and survival of pericytes in the retina have been shown to increase retinal blood vessel diameter.19) Another possible mechanism is the involvement of angioptietin-1 and its receptor Tie-2. During the critical early developmental window, angiopoietin-1 regulates the size of blood vessels; this effect is largely confined to the venous side of the circulation in most organs, including the retina.2) To clarify the mechanism of central vein enlargement, it will be important to determine whether abnormalities of pericyte recruitment and survival, and the angiopoietin-1 and its receptor Tie-2 system contribute to the observed findings.

There are some similarities in retinal vascular development...
and patterning abnormalities between newborn mice treated with VEGFR inhibitors (axitinib and KRN633) and VEGF genetically modified mice. For example, mice that only express VEGF120 exhibit severe defects in vascular growth and patterning, including a decreased number of arteries and veins. Thus, the effects of VEGFR inhibitors on retinal vascular development and patterning are likely to be mediated mainly through an attenuation of VEGF-mediated signals. In this study, VEGF-mediated signaling was disrupted shortly after birth. Therefore, these results suggest that a critical period for determining retinal vascular patterning occurs at the earliest stages of retinal vascular development, and a short-term impairment of VEGF signaling during this period may be sufficient to induce abnormal vascular patterning.

The present study showed that axitinib produces similar abnormalities in retinal vascular growth and patterns to those observed in KRN633-treated pups. However, slight differences in the degree and duration of the abnormalities were found. For example, retinal vasculature formation was delayed in both axitinib (5 mg/kg)- and KRN633 (5 mg/kg)-treated pups; however, the times at which the vasculature reached the peripheral edge of the retinal surface were different (axitinib, P12 vs. KRN633, P14). Thus, it is likely that the duration of effect of axitinib in pups after subcutaneous administration is shorter than that of KRN633, and the degree of retinal vascular growth delay affects the severity of vascular abnormalities. Our preliminary data suggest that axitinib exerts more potent anti-angiogenic effects than KRN633 in mice, because axitinib was more potent than KRN633 in inhibiting the retinal vascular formation from P4 to P6 in mice (data not shown). Therefore, the difference in the duration of drug effects is possibly due to the difference in the pharmacokinetics between the drugs.

A short-term disruption to VEGF-mediated signaling in newborn rats has been shown to disrupt postnatal alveolarization and pulmonary vascular development. A long-term impact on lung structure into adulthood and increased risk for adult cardiopulmonary disease was also found. The present study has shown that impaired VEGF signaling during critical periods of retinal vascular development can affect the architecture of the retinal vasculature. If these abnormalities persist into adulthood, they could increase the risk of impaired visual function and hasten the onset of retinal vascular disease. Further studies are needed to clarify the mechanisms underlying abnormalities of retinal vascular patterning in VEGFR inhibitor-treated mice and to determine whether abnormal vascular architecture persists into adulthood.

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