Neutralizing VEGF Decreases Tortuosity and Alters Endothelial Cell Division Orientation in Arterioles and Veins in a Rat Model of ROP: Relevance to Plus Disease

M. Elizabeth Hartnett,1,2 David Martiniuk,1 Grace Byfield,1 Pete Geisen,1 Gefei Zeng,2,3 and Victoria L. Bautch2,3

PURPOSE. To study the effects of vascular endothelial growth factor (VEGF) on endothelial nitric oxide synthetase (eNOS) and retinal vascular tortuosity and cleavage planes in a rat model of retinopathy of prematurity (ROP).

METHODS. Within 4 hours of birth, pups and mothers were cycled between 50% and 10% oxygen daily. At postnatal day (p)12, pups received either intravitreous anti-VEGF or control nonimmune rat IgG in one eye and returned to oxygen cycling until p14 when they were placed in room air (RA) for 4 days (50/10 oxygen-induced retinopathy [50/10 OIR]). Tortuosity indices and endothelial cleavage plane angles relative to the long axes of the major retinal vessels during anaphase were calculated from phosphohistone- and Alexa-isolectin–stained retinal flatmounts. Some retinas were processed for eNOS protein or phosphorylated/total eNOS.

RESULTS. Retinas from 50/10 OIR had increased tortuosity over time with peaks at p12 and p14 (P < 0.001 vs. RA) before the development of intravitreous neovascularization, which peaked at p18. Compared with RA, eNOS/actin in 50/10 OIR retinas was increased at p12 (P = 0.0005) and p14 (P = 0.047). Inhibition of VEGF with a neutralizing antibody decreased tortuosity and caused endothelial mitosis cleavage planes to orient in favor of vessel elongation but did not affect eNOS protein or activation.

CONCLUSIONS. In the 50/10 OIR model, a model with relevance to ROP, arteriolar tortuosity, and venous dilation are increased through VEGF, which influences the orientation of endothelial cell cleavage in major arterioles and veins, independent of eNOS. (Invest Ophthalmol Vis Sci. 2008;49:3107–3114) DOI: 10.1167/iovs.08-1780

-plus disease, manifest by dilation and tortuosity of retinal vessels, is an important feature of severe retinopathy of prematurity (ROP) and is predictive of poor outcome and vision loss in preterm infants.1 However, the causes of plus disease are incompletely understood. Better understanding of the mechanisms of plus disease may lead to earlier treatments or prevention of severe ROP.

Early investigators proposed that retinal vascular dilation and tortuosity in plus disease were a result of midperipheral mesenchymal mesenchymal shunting and increased retinal blood flow.2 However, studies using color Doppler imaging to measure blood flow in the central retinal artery were in disagreement. In one study, there were no significant flow differences between preterm infants with and without ROP, and in infants with ROP, there were no flow differences between those with and without plus disease.3 In another study, there was reduced blood flow in infants with plus disease.4 Neither study showed increased blood flow as initially hypothesized. However, measurements of blood flow within the central retinal artery are difficult in infant eyes and may not reflect that in the mesenchymal shunt or peripheral vessels.

Increased blood flow increases shear stress, the in-plane frictional force, on endothelial cells within blood vessels. In tortuous vessels, the acutely curved part of the vessel is believed to have greater shear stress and the opposite side reduced shear stress. Shear stress can activate endothelial nitric oxide synthetase (eNOS) to produce nitric oxide (NO), which is important in vessel homeostasis.5–7 One outcome of NO is vascular muscle relaxation and vessel dilatation, which is a feature of plus disease in ROP.

Besides shear stress, other stimuli, including hypoxia and growth factors like vascular endothelial growth factor (VEGF) can increase eNOS expression.8 Hypoxia has been associated with arterial tortuosity after middle cerebral artery occlusion,9 and when hypoxia is chronic, tortuosity is believed to be a form of angiogenesis through vessel lengthening. VEGF is mechanistically involved in the intravitreous neovascularization that occurs in animal models of ROP,10,11 and VEGF RNA was increased in the retina in a human infant with ROP.12 VEGF induces blood flow to ischemic myocardium by increasing collateral vessel formation,13 and VEGF also increases the size of capillaries during remodeling.14 VEGF is ineffective at improving angiogenesis in enos−/− mice in a murine ischemic limb model, suggesting that eNOS may be a downstream mediator of VEGF.15 Finally, eNOS was shown to be involved in hyperoxia-induced vaso-obliteration and angiogenesis in an enos−/− mouse model.16 In this last study, VEGF was not found to be downstream of eNOS. Therefore, eNOS can be activated by increased blood flow and hypoxia and may be a downstream mediator of VEGF-induced angiogenesis in some tissues.

We asked whether plus disease is in part related to increased VEGF bioactivity and, if so, whether eNOS is involved. We used the rat 50/10 oxygen-induced retinopathy (50/10 OIR) model, which develops peripheral avascular retina, intravitreous neovascularization, and arteriolar tortuosity similar to human ROP.17,18 We also measured the orientation of the cleavage of mitotic endothelial cells during anaphase19 within newly formed intraretinal arterioles and veins of pups in the 50/10 OIR model after treatment with a neutralizing antibody to VEGF as a method of assessing the effect of VEGF on dilation and tortuosity of large retinal vessels.
MATERIALS AND METHODS

Animal Treatments

All animals were cared for in accordance with the Institute for Laboratory Animal Research (Guide for the Care and Use of Laboratory Animals) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Rat Model of Oxygen-Induced Retinopathy (50/10 OIR Model)

An oxygen cycler (OxyCycler; Biospherix, New York, NY), which regulates the atmosphere inside an incubator by injecting either nitrogen or oxygen, was used to induce oxygen-induced retinopathy in newborn Sprague-Dawley rats (Charles River, Wilmington, MA). Within a few hours of birth, pups designated postnatal age (p)0 and their mothers were placed in the incubator. Oxygen was cycled between 50% and 10% every 24 hours for 14 days, and then the pups were returned to room air (RA) for 4 additional days (50/10 OIR model). These oxygen extremes were similar to oxygen saturation measurements in a preterm infant in whom severe ROP develops. In addition, in the rat, inspired oxygen has been directly related to blood oxygen level (Pao2). Litters of 12 to 14 pups were used in all experiments. Carbon dioxide in the cage was monitored and flushed from the system by maintaining sufficient gas flow.

Neutralizing Antibody to VEGF as Intraocular Injection

In each litter, half of the pups received an intraocular injection of a rat neutralizing antibody against VEGF164 (50 ng in 1μL; R&D Systems, Minneapolis, MN), in one eye and half of the litter received an intraocular injection of 50 ng rat nonimmune IgG in one eye as the control. We chose the dose that we have reported to be effective in reducing intravitreous neovascularization in the 50/10 OIR model. The fellow eyes were not injected. All intraocular injections were performed with a 33-gauge needle inserted just behind the limbus, as previously described. Litters of 12 to 14 pups were used in all experiments. Carbon dioxide in the cage was monitored and flushed from the system by maintaining sufficient gas flow.

Dissecting Retinal Tissue for Flatmounting

The pupils were heavily anesthetized by intraperitoneal (IP) injection of ketamine (2.5 mg/kg) and xylazine (1 mg/kg). Periformaldehyde (PFA; 0.7–1.0 mL, 0.5%) was directly perfused into the left ventricle before euthanatization by intracardiac injection of pentobarbital (80 mg/kg). Both eyes were enucleated, and whole eyes were fixed in 2% PFA for 2 hours. The retinas were carefully removed by using a modification of the method of Chan-Ling et al. Briefly, under a dissecting microscope an incision was made at the limbus, and the cornea was circumcised from the sclera. The lens was gently removed without disturbing the retina. The remaining eye cup was transferred to PBS, and the full extent of the retina with the ora serrata intact, was cased from the sclera using fine forceps. Care was taken to remove the hyaloidal vessels and any remaining vitreous. The retina was then placed onto a microscope slide and flattened by making four incisions, each 90° vessel in pixels over the straight distance from end to end.

Western Blot Analysis

Protein Extraction, Immunoprecipitation, and ELISA

Freshly dissected unfixed retinal tissue immersed in RIPA buffer was placed on ice for 10 minutes. The tissue was homogenized, and lysates were centrifuged at maximum speed for 10 minutes at 4°C. The supernatants were collected, and total protein in the cell lysate was determined by the BCA (bicinchoninic acid) protein assay (Pierce). The immune complexes were captured by incubation with protein G sepharose beads (GE Healthcare, Stockholm, Sweden) at 4°C for 1 hour, and the collected beads were washed three times in RIPA buffer and centrifuged at 3000g for 2 minutes at 4°C between washes. The bound protein–bead complexes were eluted with sample buffer, boiled for 10 minutes, and separated by sodium dodecyl sulfate-poly-
acrylamide gel electrophoresis (SDS-PAGE). After transfer to polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA), according to standard protocols, the blots were blocked in 5% BSA/TBST for 1 hour at room temperature, then incubated in phospho-serine antibody (1:1000, AB1604; Chemicon, Temecula, CA) overnight with gentle agitation at 4°C. Membranes were then stripped with Western blot stripping buffer (Restore Plus; 46430; Pierce Biotechnology) according to the manufacturer’s protocol and reprobed with eNOS antibody (1:1000; Santa Cruz Biotechnology). For total eNOS or β-actin analysis, 50 μg of total protein from cell lysate supernatants were diluted in sample buffer, boiled, and run for SDS-PAGE as just described. The blocked membranes were incubated with gentle agitation in either eNOS antibody (1:1000; Santa Cruz Biotechnology) overnight at 4°C or β-actin-HP antibody (1:20000; AbCam, Cambridge, MA) for 1 hour at room temperature. After four washes, the blots were incubated 1 hour with horseradish peroxidase (HRP)-conjugated secondary antibody and washed with Tris-buffered saline with Tween-20 (TBST). Visualization was performed using the enhanced chemiluminescence (Pierce Biotechnology). The signal intensity was quantified using analysis software (UN-SCAN-IT ver.6.1; Silk Scientific, Orem, UT). Rat aorta was used as a positive control.

Statistical Analysis

For Western blot experiments based on time point of analysis, the data were analyzed by Student’s t-test. In cases in which more than two treatment groups were analyzed, an overall analysis of variance (ANOVA) was performed with post hoc protected Bonferroni t-tests (SPSS ver. 14; SPSS, Chicago, IL). In analyses in which the distribution of outcomes within treatment and control groups was tested, the χ² test was used. For all comparisons, an α level of <0.05 was used as the criterion of significance.

RESULTS

Increased Arteriolar Tortuosity in the 50/10 OIR Model

Compared with RA control animals, retinal arterioles in rats in the 50/10 OIR model showed a tendency toward increased tortuosity indices after p6. There was some variability in the tortuosity index based on whether pups had just been exposed to hypoxia (even postnatal days followed the 0% O₂ cycle) or hyperoxia (odd postnatal days followed the 50% O₂ cycle), with a trend toward greater tortuosity after hypoxia. Compared with RA control animals, the tortuosity index was significantly greater in the 50/10 OIR model at p12 after hypoxia, and at p14 after 2 days in RA (Fig. 1 ANOVA, P < 0.001, post hoc protected t-test P < 0.001). In addition, the tortuosity indices at p12 and p14 were significantly greater than those in the 50/10 OIR at p7, p11, and p18. There was no difference in venous tortuosity index in the 50/10 OIR compared with RA control eyes or over time in the 50/10 OIR model (data not shown).

Increased Tortuosity Associated with Increased eNOS but Not Activated eNOS in 50/10 OIR

We and others previously found that retinal VEGF protein was increased in the 50/10 OIR model compared with RA controls at p12, p14, and at p18, the time of maximum intravitreal neovascularization, and that the peak concentration of VEGF occurred at p14.10,11 Because VEGF or blood flow induce shear stress, or both can increase eNOS expression and activation in other tissues,3-7 we measured eNOS and phosphorylation of eNOS at p12 and p14, time points when the tortuosity index was also significantly greater in the 50/10 OIR model compared with RA (Fig. 1). We found that eNOS/actin was increased at p12 (P = 0.0003; t-test) and p14 (P = 0.047), compared with RA (t-test; Fig. 2). We found no difference in eNOS/actin at p18 when the tortuosity index was reduced and did not find activation of eNOS determined by measuring phosphorylated eNOS/total eNOS at p12 (data not shown).

VEGF Causes Tortuosity in the 50/10 OIR Model

To study the possible effects of VEGF on the development of tortuosity, we compared eyes treated with a 50-ng dose of a neutralizing antibody to VEGF, which we had found to be effective in inhibiting intravitreal neovascularization in the 50/10 OIR model, with IgG-treated control eyes.11 The tortuosity index was significantly decreased at p14 in retinal vessels after a single dose of 50 ng neutralizing VEGF antibody given at p12 (Fig. 3), but the effect was not present at p18 (data not shown). In part, this may have been because retinal tortuosity became less in the 50/10 OIR model by p18 (Fig. 1).

Effect of Neutralizing VEGF with Antibody on eNOS

Investigators have reported eNOS to be a downstream mediator of VEGF-induced angiogenesis in other tissues.15 Since increased eNOS protein was associated with the time points of greatest tortuosity and neutralizing the bioactivity of VEGF with an antibody to VEGF reduced tortuosity in the 50/10 OIR model, we wanted to determine whether inhibiting VEGF with a neutralizing antibody would reduce eNOS. We found that 50 ng of a neutralizing antibody to VEGF did not change
eNOS concentration relative to actin (Fig. 4) or phosphorylated/total eNOS at p14 (Fig. 5).

Effect of VEGF on Orientation of Endothelial Cell Cleavage Planes

We have shown in a prior study that increased signaling through VEGF receptor 2 (VEGFR2) in an \textit{flt-1}\textsuperscript{-/-}/H9252 embryonic stem cell model, a model lacking blood flow, results in more randomly oriented endothelial cell cleavage planes, measured during the anaphase, compared with the control, and this pattern is rescued with a soluble \textit{flt-1} transgene linked to the PECAM promoter/enhancer.\textsuperscript{19} We have also shown that inhibition of the bioactivity of VEGF with a neutralizing antibody reduces intraretinal signaling through VEGFR2 in the 50/10 OIR model, particularly in the inner vascular plexus.\textsuperscript{11} We wanted to determine whether endothelial cleavage plane orientation was altered in the large vessels in the 50/10 OIR model, favoring dilation and tortuosity such as that in human plus disease and whether reducing VEGFR2 signaling with a neutralizing antibody to VEGF would restore the orientation of endothelial cell divisions such as that in normal development in RA. The angle between the cleavage plane of phosphohistone-stained mitotic figures determined during the anaphase and vessel long axis was determined in major vessels within the retinas of RA-raised and in VEGF antibody–treated, control IgG–treated, and noninjected eyes (Figs. 6E, 6F).

In RA-raised rats, the total number of mitoses identified per retina decreased with increasing postnatal age (38.83 at p6, 0 at p14; Table 1), with veins having more mitoses than arterioles. When only analyzing mitotic figures in the anaphase, there were few cleavage planes in both arterioles and veins in RA-raised rats. The prevalence of cleavage planes at 80° to 90° during the anaphase, compared with the control, and this pattern is rescued with a soluble \textit{flt-1} transgene linked to the PECAM promoter/enhancer.\textsuperscript{19} We have also shown that inhibition of the bioactivity of VEGF with a neutralizing antibody reduces intraretinal signaling through VEGFR2 in the 50/10 OIR model, particularly in the inner vascular plexus.\textsuperscript{11} We wanted to determine whether endothelial cleavage plane orientation was altered in the large vessels in the 50/10 OIR model, favoring dilation and tortuosity such as that in human plus disease and whether reducing VEGFR2 signaling with a neutralizing antibody to VEGF would restore the orientation of endothelial cell divisions such as that in normal development in RA. The angle between the cleavage plane of phosphohistone-stained mitotic figures determined during the anaphase and vessel long axis was determined in major vessels within the retinas of RA-raised and in VEGF antibody–treated, control IgG–treated, and noninjected eyes (Figs. 6E, 6F).

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Figure 6. (A) Arteriolar and (B) venous cleavage planes in normal retinal vascular development at p5, p6, and p11. By p14 and p15 when development of the inner retinal vascular plexus is complete, no mitosis planes were found in each of five retinas surveyed. (C) Angle of arteriole and (D) venous retinal endothelial cell division planes, grouped by degrees from the long axis of the vessel, in 50/10 OIR pups injected with either 50 ng VEGFab or 50 ng control IgG. A value of 90° is perpendicular to the vessel long axis, and 0° is parallel to the vessel long axis. Treatment with neutralizing antibody to VEGF caused veins to have fewer cleavage planes that were between 0° and 20° relative to the long axis of the vessel compared to IgG injected controls (P < 0.05, \( \chi^2 \)). These data provide support that treatment with antibody to VEGF reduces venous dilation. (E) Retinal flatmount from rat pup in 50/10 OIR treated with 50 ng intravitreous VEGFab and labeled with isolectin for retinal vessels and phosphorylase to stain mitoses. Angle between mitotic cleavage plane of dividing endothelial cell and long axis of the vessel measures 7.57°. (F) Flatmount of retina from an eye injected with 50 ng VEGFab showing a mitotic cleavage angle of 84.09°.
from the vessel long axis (favoring vessel elongation) increased with age of development in both arterioles and veins (Figs. 6A, 6B).

From two litters of rats raised in the 50/10 OIR model, pups received an intravitreal injection of either 50 ng neutralizing antibody to VEGF or IgG in one eye at p12 and were analyzed at p14. There were more mitotic events in the 50/10 OIR model than RA at p14 (Table 1). Within the 50/10 OIR model, there were slightly fewer mitoses, although not significantly so, in eyes that had received the neutralizing antibody to VEGF compared to IgG (Table 1).

We found that arterioles of pups treated with intravitreal injections of neutralizing antibody to VEGF appeared to have a greater number of cleavage planes in anaphase that were between 80° and 90°, relative to the long axis of the vessel compared with control IgG treated eyes (Fig. 6C). This orientation, which causes elongation of the retinal vessel, corresponded more closely to that in normal development in RA-raised pups, in which fewer cleavage planes were found, but most occurred at the axis 70° to 90° from the long axis of the developing vessel19 (Fig. 6A). Furthermore, veins treated with VEGF antibody had fewer cleavage planes that were between 0° and 20° relative to the long axis of the vessel, compared with veins in IgG-injected control eyes (P < 0.05, \( \chi^2 \); Fig. 6D). This result suggests that treatment with antibody to VEGF reduces venous dilation, a feature present in human plus disease.

### DISCUSSION

We found, in agreement with other investigators, that arteriolar tortuosity was increased in the rat 50/10 OIR model of ROP compared with RA-raised rats.17 We had previously measured retinal VEGF protein in the 50/10 OIR model and found significantly greater protein at p12, p14, and p18 compared with RA, with the peak concentration of retinal VEGF occurring at p14.10,11 The p12 and p14 time points also correspond to the time points with significantly higher tortuosity indices in the 50/10 OIR model compared with the RA control. Furthermore, retinal eNOS was increased at p12 and p14 compared with the RA control at the same time points that tortuosity and VEGF were increased, and these time points preceded the development of intravitreal neovascularization.10,11 However, although eNOS has been shown to be downstream of VEGF signaling in other tissues,8 we were unable to provide evidence that inhibition of VEGF with a neutralizing antibody, effective at reducing tortuosity and intravitreal neovascularization in the 50/10 OIR model,11 had an effect on eNOS concentration or activation. We interpret these data to mean that the effects of VEGF on tortuosity are independent or downstream of eNOS. Brooks et al.16 found that in enos/−/− mice, hypoxia-induced vasoobliteration was less severe, but that VEGF concentration was not affected and did not appear to be downstream of eNOS signaling. Beauchamp et al.13 found, also using the mouse OIR model, that NO had opposing effects on vaso-obliteration and VEGFR2 expression that depended on the redox state of the retina. Both these studies used constant high oxygen exposure in the mouse OIR model, which differs from the fluctuations and oxygen extremes used in the rat 50/10 OIR model. Based on our data and that in the literature, we speculate that the increase in eNOS seen by Western blot at p12 and p14 in the 50/10 OIR model may be related to other factors.24-25 including hypoxia and increased shear stress,8 and not directly to VEGF. Although previous studies, failed to show increased blood flow in human ROP by Doppler imaging,3,4 the techniques are difficult in infants. Furthermore, blood flow-induced shear stress in a tortuous vessel is complex. Whereas, in a straight vessel, shear stress is increased throughout the inner circumference of the vessel when blood flow increases,26 in tortuous vessels and at branch points, the relationships within vessels and the signaling within endothelial cells are changed.26 Blood flow measurements and shear stress may vary at different regions within the tortuous vessel. Therefore, it is conceivable that increased shear stress may occur in tortuous vessels and activate eNOS without registering an increase in blood flow measurements.

We inhibited VEGF with a neutralizing antibody at a concentration that we had found to reduce intravitreal neovascularization and intraretinal VEGFR2 signaling.11 We found that arteriolar tortuosity was reduced, and the orientation of endothelial cell cleavage planes during the anaphase of the major veins and arterioles within the vascularized retina was changed. In veins, treatment with VEGF antibody caused a change from a parallel orientation of cleavage planes to the vessel long axis favoring vessel widening to one in which cleavage planes were perpendicular to the vessel long axis favoring vessel elongation. In arterioles, treatment with VEGF antibody caused a change from a parallel orientation of cleavage planes to the vessel long axis favoring vessel elongation. In arterioles, treatment with VEGF antibody caused a change from a parallel orientation of cleavage planes to the vessel long axis favoring vessel widening to one in which cleavage planes were perpendicular to the vessel long axis favoring vessel elongation. In arterioles, treatment with VEGF antibody caused a change from a parallel orientation of cleavage planes to the vessel long axis favoring vessel elongation. In arterioles, treatment with VEGF antibody caused a change from a parallel orientation of cleavage planes to the vessel long axis favoring vessel widening to one in which cleavage planes were perpendicular to the vessel long axis favoring vessel elongation. In arterioles, treatment with VEGF antibody caused a change from a parallel orientation of cleavage planes to the vessel long axis favoring vessel widening to one in which cleavage planes were perpendicular to the vessel long axis favoring vessel elongation.
means to induce a gain in VEGF signaling through VEGFR2. The data from the embryonic stem cell model demonstrated that the orientation of daughter cell divisions is necessary in blood vessel morphogenesis and is regulated by signaling through VEGFR2 in a flow-independent manner.19 These findings contrasted with previous ones that proposed that shear stress is necessary and plays a major role in the morphogenesis of blood vessels.29 Thus, it appears that shear stress is not necessary for regulating the orientation of daughter cell cleavage planes in developing tissue. In our present study, we found that VEGF was important in the orientation of cleavage planes of endothelial cells, this time in recently developed major vessels in retinas with blood flow. However, it appeared that although eNOS, activated by shear stress,25,26 was increased in the 50/10 OIR model, it was not involved in VEGF induced tortuosity and dilation. Although remodeling of capillaries during development in the rat eye has been carefully described,30 our findings suggest that already-developed major intraretinal vessels, not just the newly formed capillaries, have endothelial divisions that are influenced by VEGF concentration. The importance of VEGF in human retinal and iris vessel dilatation and engorgement has been reported in a case series of human infants with aggressive posterior ROP, who experienced reduction in vessel tortuosity and dilation after treatment with bevacizumab, a monoclonal antibody against VEGF.31

VEGF is known to affect vascular development by influencing several events, including the number of endothelial tip cells to stalk cells,32 the direction in which filopodia point,33 and endothelial cell proliferation and migration.34 Once vessels have formed, how VEGF alters vessel diameter or tortuosity is largely unknown. However, based on development in other tissues, we know the orientation of the cleavage plane in dividing cells is regulated, in part, by the actin cytoskeleton.29 Although the major vessels in the retina have already developed, our data show that mitoses are ongoing at least through p14 in the 50/10 OIR model. VEGF has been shown to induce actin cytoskeletal changes through signaling through integrins35 or through activation of Rho GTPases,36 for example, and through such cytoskeletal events, it is possible that the orientation of the cleavage planes during anaphase are altered.

In summary, our findings provide support that the causes of vascular tortuosity and dilation in the 50/10 OIR model, relevant to human ROP, are from increased VEGF signaling through VEGFR2,19 based on previous studies in which the same model, means of inhibition of VEGF, and dose were used.11 Our data support that VEGF is an early event in the development of tortuosity and dilation. Although eNOS expression is increased in the 50/10 OIR model, our data do not support eNOS as downstream of VEGF-induced tortuosity and dilation in the 50/10 OIR model of ROP.

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