Time-Lapse Imaging of Retinal Angiogenesis Reveals Decreased Development and Progression of Neovascular Sprouting by Anecortave Desacetate

Noriyuki Unoki, Tomoaki Murakami, Ken Ogino, Masayuki Nukada, and Nagahisa Yoshimura

Purpose. To elucidate the effects of anecortave desacetate (AD) treatment on the kinetics of neovascular sprouting and its molecular mechanisms in retinal explants and during retinal vascular development in mice.

Methods. The antiangiogenic effects of AD on retinal angiogenesis were evaluated using two different models: a retinal explant model treated with vascular endothelial growth factor (VEGF) and a mouse model of postnatal retinal vascular development. Time-sequential observation was followed by the quantification of movements in neovascular sprouts and microglia. Real time-PCR was performed for the measurement of mRNA levels.

Results. AD treatment significantly reduced the number of neovascular sprouts in retinal explants in a dose-dependent manner. Time-lapse imaging demonstrated that AD suppressed the new development and elongation of neovascular sprouts and the motility of tip cells. Moreover, AD treatment disturbed the filopodial extension and significantly decreased the transcriptional levels of KDR and platelet-derived growth factor-B, which are highly expressed in tip cells. In addition, it was confirmed that AD inhibited postnatal development of the retinal vasculature in mice, including filopodial extension in tip cells. These data suggest that AD suppresses both the development and the progression of sprouting angiogenesis. Interestingly, VEGF-induced microglial movements were also reduced in the retinal explants treated with AD. These changes were consistent with decreased mRNA levels of SDF-1 and Flt-1, which regulate the activation of inflammatory cells.

Conclusions. AD inhibited both the development and the progression of VEGF-induced retinal angiogenesis mediated, in part, by the suppression of tip cell motility in two angiogenic models. (*Invest Ophthalmol Vis Sci.* 2010;51:2347–2355) DOI: 10.1167/iovs.09-4158

Neovascular complications in retinal vascular diseases, including diabetic retinopathy and age-related macular degeneration, often cause severe visual loss. Recent advances have elucidated that multiple steps of the angiogenic cascade involve several cell types directed by a variety of growth factors.[1–5] Among these molecules, crucial factors, such as vascular endothelial growth factor (VEGF), are assumed to be therapeutic targets, and several clinical trials have been performed in diseases associated with pathologic angiogenesis, including cancer and posterior segment diseases.[6,7] Folkman et al.[8] reported a novel class of steroids, angiostatic steroids, that inhibit angiogenesis but that do not have glucocorticoid (anti-inflammatory) or mineralocorticoid activities. Other investigators[9–11] have demonstrated their antiangiogenic effects in several animal models. Anecortave acetate (AA; Retaane, 15 mg [anecortave acetate suspension]; Alcon, Fort Worth, TX) is a novel, angiostatic cortisene that inhibits pathologic ocular angiogenesis and does not exhibit typical ocular glucocorticoid-induced side effects, such as cataract formation and increased intraocular pressure potentially leading to glaucoma. To eliminate the untoward glucocorticoid effects and maintain the angiostatic activity of AA, a hydroxyl group in cortisol was replaced by a double bond at the C9–11 position, and a 21-acetate was added to promote drug penetration and increase the duration of action.[12] AA has one major active metabolite, anecortave desacetate (AD), that is formed by deacetylation.[13] In various preclinical models, AA has been shown to suppress the expression of some extracellular proteinases,[10] the proliferation and differentiation of endothelial cells, and the synthesis of proangiogenic growth factors and their receptors.[11] Preclinical efficacy pharmacology studies demonstrate that AA can significantly inhibit corneal, retinal, and choroidal neovascularization as well as tumor growth in various species.[10,11,14,15] Of note, in a rat model of retinopathy of prematurity (ROP), AA inhibited the growth of new pathologic vessels but did not induce an observable affect on normal retinal vascular development.[10] Genotoxicity, carcinogenicity, and reproductive toxicity studies in vivo have revealed no significant ocular or systemic toxicities.[16] However, how these individual mechanisms work in concert on the antiangiogenic effects of AA remain to be fully elucidated. Angiogenesis is a dynamic phenomenon involving the sprouting of new vessels from preexisting mature vessels. Despite some suggestive data, it is largely unknown how quiescent and mature endothelial cells achieve an activated phenotype that results in movement and proliferation.[17] For clinical applications, it is important to resolve this question because we must understand the potential adverse effects on physiological vasculature given that most receptors of growth factors are expressed in mature vessels and in angiogenic sprouting.[18] To date, only a small amount of evidence has demonstrated the kinetics of angiogenesis movement and migration of vascular cells.[19–21]

Sprouting angiogenesis involves collective migration processes.[22] Tip cells, which lead stalk cells, have receptors for attractive or repulsive cues, such as KDR and Unc5b,[23,24] and

From the Department of Ophthalmology and Visual Sciences, Kyoto University Graduate School of Medicine, Sakyo, Kyoto, Japan.

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Corresponding author: Tomoaki Murakami, Department of Ophthalmology and Visual Sciences, Kyoto University Graduate School of Medicine, 54 Shogoin-Kawaracho, Sakyo, Kyoto 606-8507, Japan; mutomo@kuhp.kyoto-u.ac.jp.

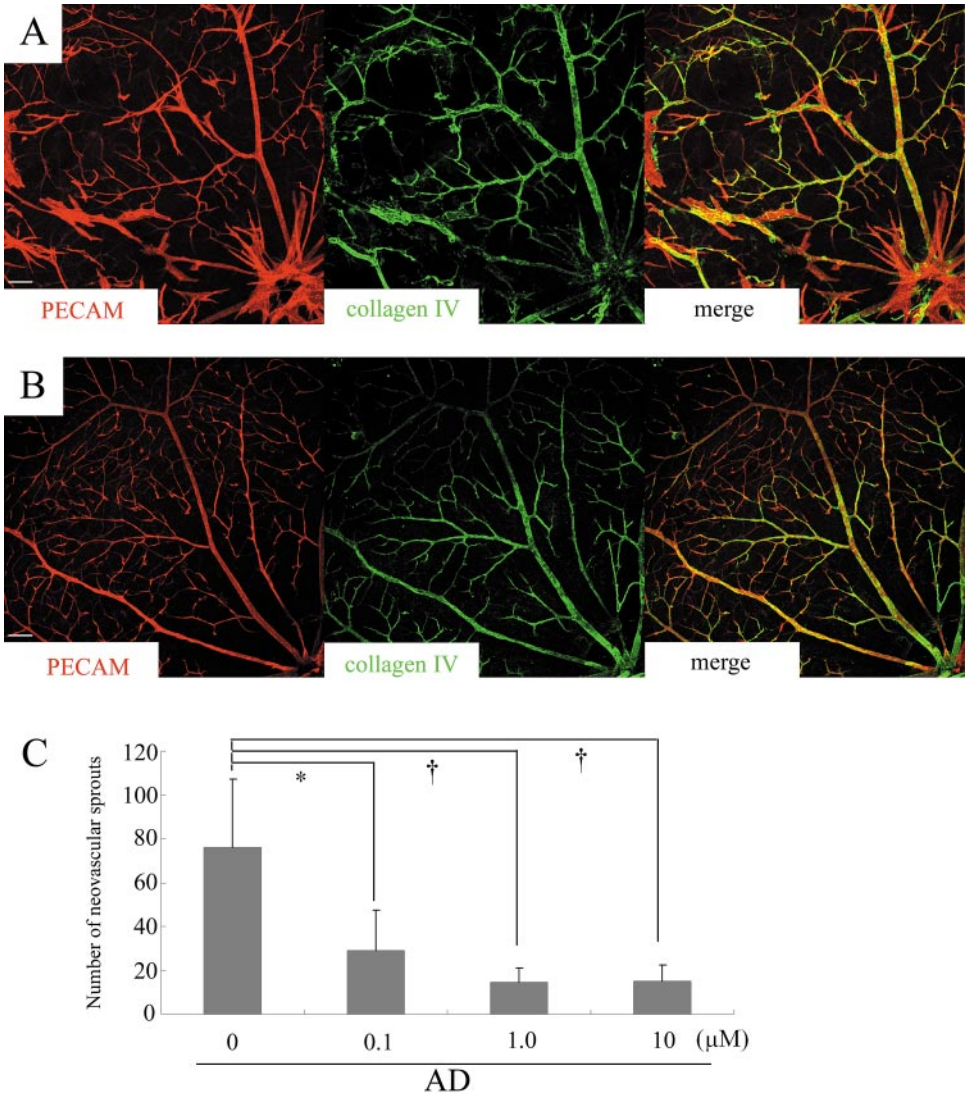

FIGURE 1. AD attenuates VEGF-induced neovascular sprouting in retinal explants. Retinal explants were treated with 25 ng/mL VEGF alone (**A**) or with VEGF + 1.0 μM AD (**B**) for 96 hours, followed by immunostaining with PECAM (**C**). Retinas were treated with the indicated doses of AD, and neovascular sprouts were quantified. $^*P < 0.05$; $\dagger P < 0.01$. Scale bar, 100 μm.

regulate the coordinated processes of neovascular sprouting. In addition, platelet-derived growth factor-B (PDGFB) is secreted by tip cells and promotes the recruitment of pericytes, which migrate along newly formed vessels.[23,25] Stromal cell-derived factor-1 (SDF-1) is a potent contributor to angiogenesis,[26] and we have recently found that tip cell motility is regulated by SDF-1 (TM, unpublished data, 2009).

In the present study, we demonstrated the kinetics of neovascular sprouts in retinal explants treated with VEGF with or without AD. Both the development and the progression of neovascular sprouts were suppressed by AD, consistent with the identified transcriptional changes in angiogenic molecules. These data suggest that AD can be expected to have both preventive and therapeutic effects in pathologic ocular angiogenesis.

METHODS

Materials

Recombinant human VEGF was purchased from R&D Systems (Minneapolis, MN). Commercially obtained antibodies against platelet and endothelial cell adhesion molecule (PECAM; BD Biosciences, San Jose, CA), type IV collagen (Chemicon, Temecula, CA), and Iba-1 (Wako, Osaka, Japan). All other reagents were purchased from Sigma (St. Louis, MO) or Wako. AD was a kind gift from Alcon Research Ltd.

Animals

All animal procedures were performed in accordance with both the guidelines for animal experiments at the Kyoto University Graduate School of Medicine and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. C57BL6/J mice were purchased from SLC Japan (Hamamatsu, Japan). For time-lapse imaging, Tie2-GFP mice were obtained from the Jackson Laboratory (Bar Harbor, ME).[27]

Retinal Explants in Mice

We performed organ cultures of adult retinas, as described previously.[21] Briefly, neural retinas isolated from 7- to 8-week-old mice were placed on a chamber filter and cultured in a six-well culture plate. We quantified the number of neovascular sprouts according to previously described methods[21] by which PECAM-positive and type IV collagen-negative cordlike or tubelike structures were defined as neovascular sprouts and were counted using a 20× objective lens.

Time-Lapse Imaging of Retinal Explants

Time-sequential images of angiogenesis were obtained in retinal explants of Tie2-GFP transgenic mice, as previously described.[21] Briefly, after 96 hours of culture, retinas were placed on a glass-bottomed dish, and confocal images were obtained at 15-minute intervals. We further evaluated the movement of neovascular sprouts and microglia from

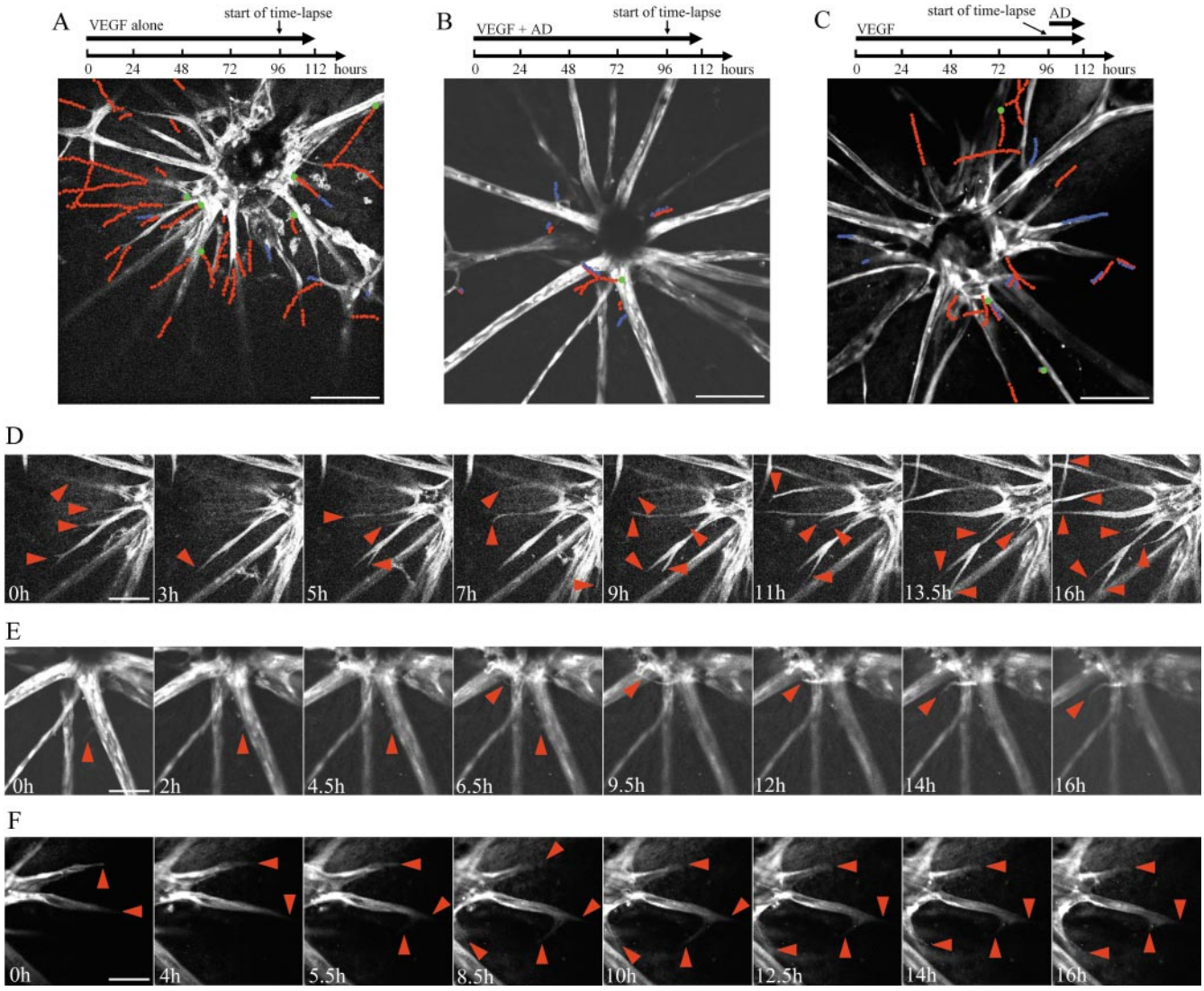

FIGURE 2. Time-lapse imaging of neovascular sprouting in retinas treated with AD. Time-lapse imaging began at 96 hours after the onset of organ culture. Both VEGF (25 ng/mL) and AD (1.0 μM) treatment attenuated both the development and elongation of neovascular sprouts (**B**, **E**), compared with VEGF alone (**A**, **D**). To evaluate the effects on sprouts, which had already developed, both VEGF and AD were added after 96 hours incubation with VEGF alone (**C**, **F**). The elongation of sprouts was reduced, and some sprouts showed the regression. VEGF developed several de novo sprouts (**A**), whereas both VEGF and AD reduced their development (**B**). Traces of leading edges highlighted in *red* (forward movement) and *blue* (backward movement). *Green dots*: newly developed sprouts (**A–C**). *Arrowheads*: neovascular sprouts. Time from the start of time-lapse imaging (**D–F**). Scale bars: 100 μm (**A–C**); 50 μm (**D–F**).

TIFF images exported into graphic editing software (Photoshop; Adobe Systems, San Jose, CA). After correction for retinal position using the optic disc, the most distal end from the parent vessel was determined in each neovascular sprout for each time point. We have defined the elongation of neovascular sprouts as the length between the distal end at the baseline and at each time point. Extension lengths of the leading edges were the differences between the distal ends in two sequential images. Positive values were given to extensions, whereas retractions received negative values. To measure microglial movements, single cells that had no connection to the retinal vasculature were evaluated after colocalization with Iba-1, a macrophage/microglial marker (TM, unpublished data, 2009). Moving distances of the microglia were defined as the differences between the centers of the cell body at two sequential time points. Morphologic changes in microglia were classified as either rounded or amoeboid. We defined amoeboid microglia as those with dynamically extended or retracted pseudopods and rounded microglia as those with a relatively homogeneous GFP signal and no pseudopod-like appearance.

For the quantification of each parameter, three neovascular sprouts or microglia were chosen in a masked fashion, and the probability distribution of the extension lengths was calculated based on all three data sets.

Real-Time PCR of Retinal Explants

Total RNA was isolated from cultured retinas with a PCR kit (RNAqueous-4PCR; Ambion, Austin, TX) according to the manufacturer's instructions. Each preparation was then applied to a cDNA synthesis reaction with a cDNA synthesis kit (First-Strand; GE Healthcare, Buckinghamshire, UK). Validated primers for KDR/Flk-1, Flt-1, neuropilin-1, PDGFB, SDF-1, and CXCR4 were obtained from Applied Biosystems (Foster City, CA). Each sample was measured by real-time PCR in triplicate experiments (ABI Prism 7000 Sequence Detection System; Applied Biosystems) under the following cycling conditions: 2 minutes at 50°C and 10 minutes at 95°C, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Expression levels were normalized to the S18 mRNA levels.

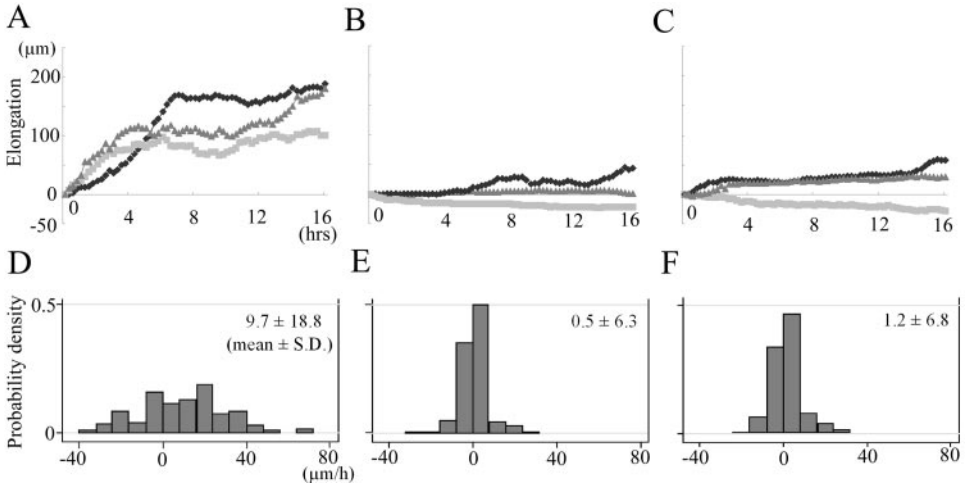

FIGURE 3. Quantification of elongation of neovascular sprouts and extension length of leading edges in tip cells. VEGF-induced neovascular sprouts elongated gradually with repeated extension and retraction of leading edges (**A**, **D**), whereas both VEGF and AD showed decreased elongation with less motility of leading edges (**B**, **E**). Retinas were treated as shown in Figure 2C, and quantification revealed that the decreased elongation or regression of neovascular sprouts that had already developed and the motility of leading edges were also suppressed (**C**, **F**). Time was measured from the start of time-lapse imaging (**A**–**C**).

Postnatal Retinal Vascular Development in Mice

AD (1.0 nmol/g body weight) was administrated intraperitoneally to randomly selected neonatal mice on postnatal day (P) 2, and littermates injected with vehicle served as controls. At 48 hours after treatment, perfusion fixation was performed, followed by retinal isolation and immunostaining. For quantification, the radius of the vascularized area was measured as described previously.[25] Briefly, the radius was determined by measuring the length between the center of the optic nerve and the edges of leading vessels (LSM Image Browser; Zeiss GmbH, Oberkochen, Germany).

Immunohistochemistry

After the indicated periods of organ culture, retinas were fixed with 4% paraformaldehyde (PFA) in PBS. For in vivo experiments, perfusion fixation using 4% PFA/PBS was performed, and the retinas were isolated. After permeation with 0.5% Triton X-100, immunostaining procedures were performed with fluorescent secondary antibodies, as described previously.[21] Images of flat-mount retinas were captured under a confocal microscope (LSM 5 Pascal; Zeiss GmbH).

Electron Microscopy

Cultured retinas were fixed with 1.2% glutaraldehyde and 2% PFA in PBS at room temperature for 2 hours, followed by the postfixation step for 2 hours with 1% osmium tetroxide in 0.1 M PBS. Fixed retinas were dehydrated by soaking in increasing ethanol concentrations (50%, 60%, 70%, 80%, 90%, 99%, 100%) and propylene oxide for 1 hour. After the incubation with 50%, 75% epoxy resin-propylene oxide mixtures, dried retinas were embedded in 100% epoxy resin and incubated at 60°C for 3 days for polymerization. Ultrathin sections measuring 1 × 1 mm (60–90 nm) and sliced with microtome (Ultracut; Leica, Wetzlar, Germany) were mounted on copper grids. Images were acquired using a transmission electron microscope (H-7650; Hitachi, Tokyo, Japan) with an acceleration voltage of 80 kV.

Statistical Analysis

The results shown are expressed as the mean ± SD, unless otherwise indicated. For statistical analysis, we used the Student's *t*-test or analysis of variance to compare quantitative data populations with normal distribution and equal variance. Data were analyzed by using the Mann-Whitney *U* rank sum test or the Kruskal-Wallis test for populations with nonnormal distributions or unequal variance. $P < 0.05$ was considered statistically significant.

RESULTS

AD Suppresses VEGF-Induced Neovascular Sprouting in Retinal Explants

We first administrated AD at the indicated doses to retinal explants treated with VEGF (25 ng/mL) for 96 hours to determine the effects of AD on retinal angiogenesis. Retinal explants incubated with VEGF alone presented PECAM-positive and type IV collagen-negative neovascular sprouts originating from PECAM-positive and type IV collagen-positive parent vessels (Fig. 1A). In contrast to VEGF-treated samples, the retinas incubated with both VEGF and AD had only a few neovascular sprouts (Fig. 1B). We quantified the number of neovascular sprouts and found that AD significantly reduced the number of neovascular sprouts in a dose-dependent manner (Fig. 1C).

AD Reduces the Development of Neovascular Sprouts

Angiogenesis is composed of multiple steps in its molecular and cellular mechanisms in which AD may impose its therapeutic effect. The development or progression of sprouting angiogenesis may be suppressed or the regression of neovascular sprouts, which have already developed, may be induced by apoptotic changes or retractive cues. To determine whether AD inhibits the development of neovascular sprouts, we obtained time-lapse imaging of retinal angiogenesis, as described in the methods section. Compared with VEGF alone, de novo development of sprouts was reduced and thinner in the retinas incubated with both 25 ng/mL VEGF and 1.0 μM AD (Figs. 2A, 2B, Movies S1, S2; all movies available at http://www.iovs.org/cgi/content/full/51/5/2347/DC1).

Elongation and Motility of Sprouts Are Attenuated by AD

We further analyzed the kinetics of neovascular sprouts to evaluate the effects of AD on the progression of sprouting angiogenesis. AD decreased the elongation of sprouts, whereas gradual increases were observed with VEGF alone (Figs. 2A, 2B, 2D, 2E, 3A, 3B; Movies S1, S2, S3, S4). When we focused on the motility of leading edges in tip cells, both extension and retraction were reduced by AD (Figs. 2A, 2B, 2D, 2E; Movies S1, S2, S3, S4). We quantified it and found that the probability distribution of the extension length presented a normal distribution with a large SD in VEGF-treated retinas that converged to zero on AD treatment (Figs. 3D, 3E).

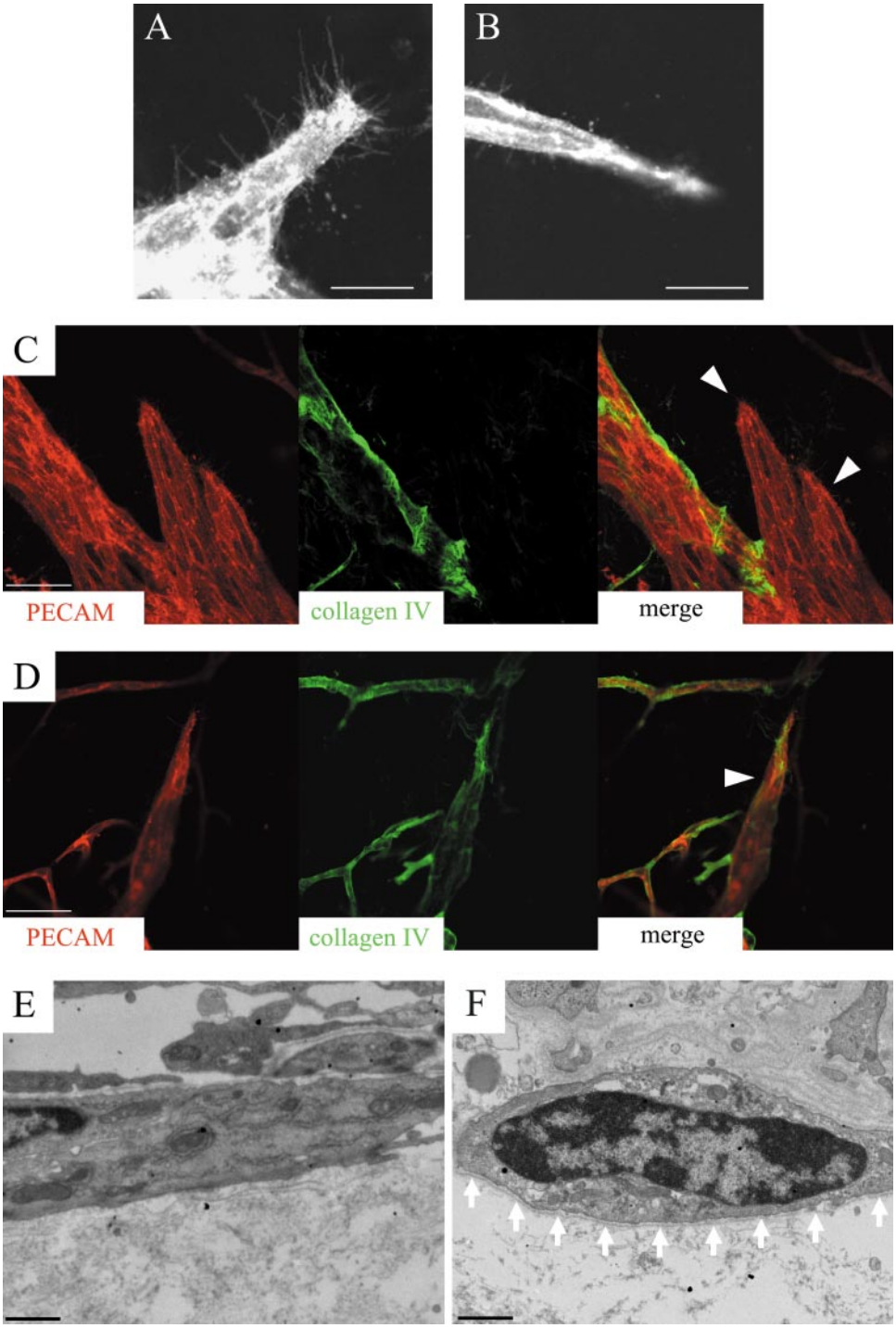

FIGURE 4. Changes in filopodial extension and basement membrane in neovascular sprouting. After 96-hour treatment with VEGF alone, retinas were treated with either VEGF + AD (**B**, **D**) or VEGF alone (**A**, **C**) for another 24 hours. Magnified images of neovascular sprouts showed filopodial extension by VEGF (**A**) that was disturbed by AD treatment (**B**). Sprouts treated with AD were covered with type IV collagen (**D**) compared with those in VEGF alone (**C**). Electron microscopic image of neovascular endothelial cells in the vitreous treated with VEGF+ AD showed thin and immature basement membrane (**F**) that was absent in those under VEGF treatment alone (**E**). Scale bars: 20 μm (**A**, **B**), 100 μm (**C**, **D**), 1 μm (**E**, **F**). (**C**, **D**, *arrowheads*) Neovascular sprout. (**F**, *arrows*) Basement membrane.

To evaluate how AD affects the movements of neovascular sprouts that had already developed, retinas were incubated with VEGF alone for 96 hours; this was followed by treatment with both VEGF and AD for another 24 hours (Fig. 2C). Time-lapse imaging demonstrated that the elongation of sprouts was reduced and that some of the developed sprouts had regressed (Figs. 2C, 2F, 3C; Movies S5, S6). With regard to the motility of tip cells, the probability distribution of the extension length indicated that both extension and retraction of leading edges were attenuated by AD treatment, in contrast to those treated with VEGF alone (Fig. 3F). These data suggest that AD inhibited the progression and development of neovascular sprouts, though we could not elucidate whether AD induced their regression Additionally, the coincidence of less motility in tip cells and less elongation of sprouts under AD treatment suggest that AD might have induced the maturation or quiescence of neovascular sprouts.

Changes in Basement Membrane and Filopodial Extension in Neovascular Sprouting

These results encouraged us to observe filopodial extension in neovascular sprouts because it represents the function of tip cells.[23] After 96-hour incubation of VEGF alone, explants were treated with both VEGF and AD for another 24 hours. Immunostaining showed that VEGF induced PECAM-positive filopodial extension in neovascular sprouts, which was distorted and re-

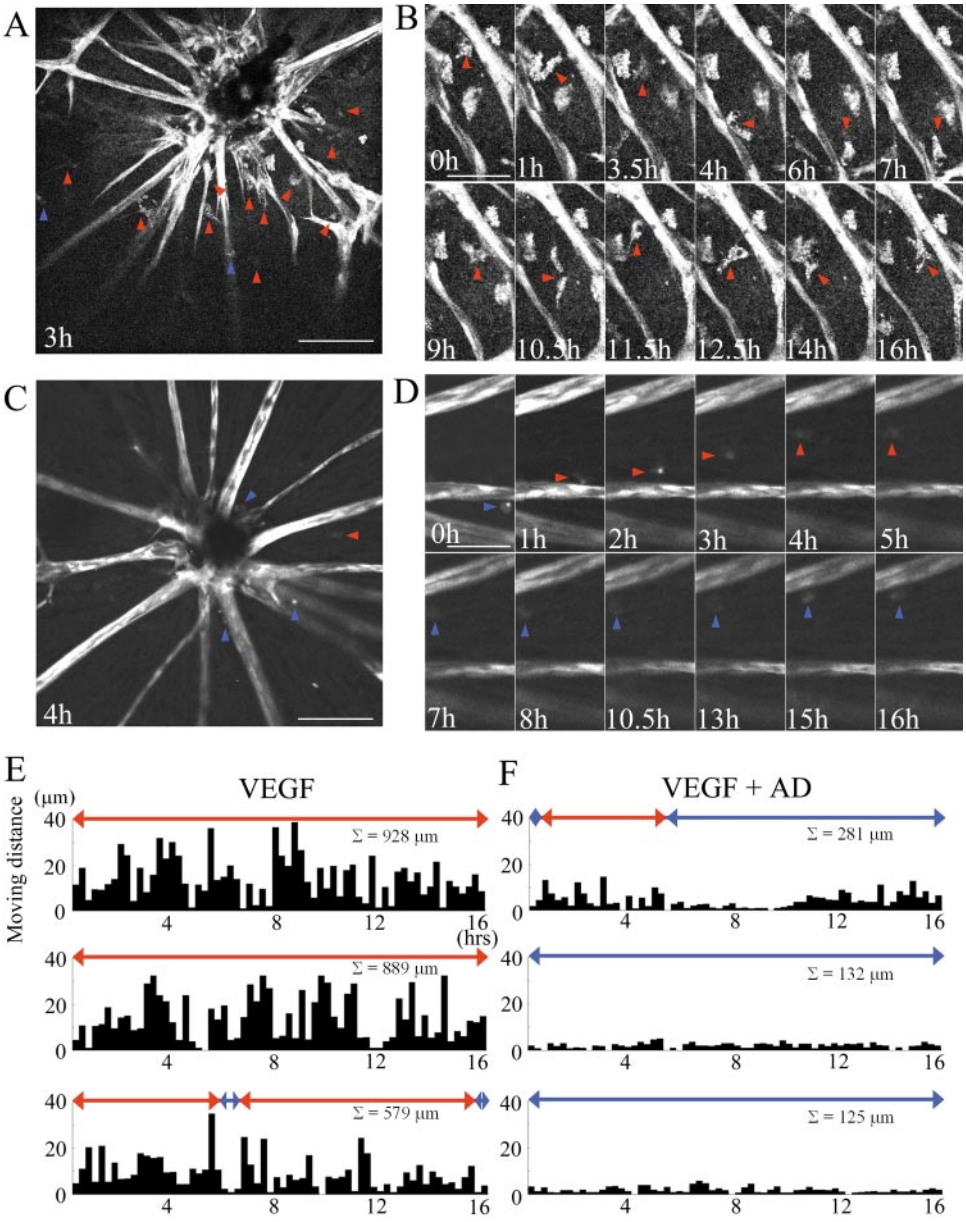

FIGURE 5. Microglial movements were suppressed with AD treatment. GFP-positive cells, which corresponded to microglia, showed amoeboid morphology and moved rapidly in the retinas treated with VEGF alone (**A**, **C**, **E**). However, both VEGF and AD treatment increased rounded and quiescent microglia (**B**, **D**, **F**). (**E**, **F**) Microglial movements were quantified. *Red arrowheads*: amoeboid microglia. *Blue arrowheads*: rounded microglia. Time from the start of time-lapse imaging was shown. Scale bars: 100 μm (**A**, **C**); 50 μm (**B**, **D**).

duced by AD treatment (Figs. 4A, 4B). This observation is compatible with the decreased motility of leading edges.

Previous publications have demonstrated that AA increases the mRNA of plasminogen activator inhibitor-1 in rat retinas,[10] which could attenuate the activities of proteolytic enzymes including matrix metalloproteinases and urokinase plasminogen activator.[11,28] These proteases degrade extracellular matrix containing vascular basement membrane (BM), which initializes the multiple steps of angiogenesis.[29] These results prompted us to investigate how AD affects the BM of neovascular sprouts. PECAM-positive cells in sprouts of retinas treated with VEGF alone were not covered with type IV collagen, whereas AD treatment resulted in sprouts covered with type IV collagen (Figs. 4C, 4D). Furthermore, electron microscopy showed thin and immature BM around neovascular endothelial cells sprouting into the vitreous treated with both VEGF and AD for the last 24 hours (Fig. 4F) compared with the absence of BM in those under VEGF treatment alone (Fig. 4E). These findings suggest that AD might enable neovascular sprouts to mature to some extent as part of its antiangiogenic effects.[30]

Microglial Motility Is Decreased by AD

Because recent advances have proved that microglia modulate retinal angiogenesis,[31,32] we also evaluated the movements of microglia in retinal explants. We characterized the GFP-positive cells in retinas of Tie2-GFP mice and found that the isolated single GFP-positive cells containing dotlike GFP signal were stained with a macrophage/microglial marker, Iba-1 (data not shown). We also analyzed in vivo retinas isolated from adult mice. Immunostaining demonstrated that most Iba-1–positive cells reside in extravascular spaces with ramified morphology, suggesting that these cells are primarily microglial cells (data not shown).

We then evaluated those single GFP-positive cells in time-sequential images and found that VEGF induced rapid movements in these cells, whereas AD partially attenuated their motility (Fig. 5; Movies S1, S2, S7, S8). Interestingly, the rapidly moving cells in VEGF-treated retinas were amoeboid like, though AD increased the round microglia that were less motile (Fig. 5; Movies S1, S2, S7, S8). During the activation of microglia, cell shapes were changed from ramified and round to

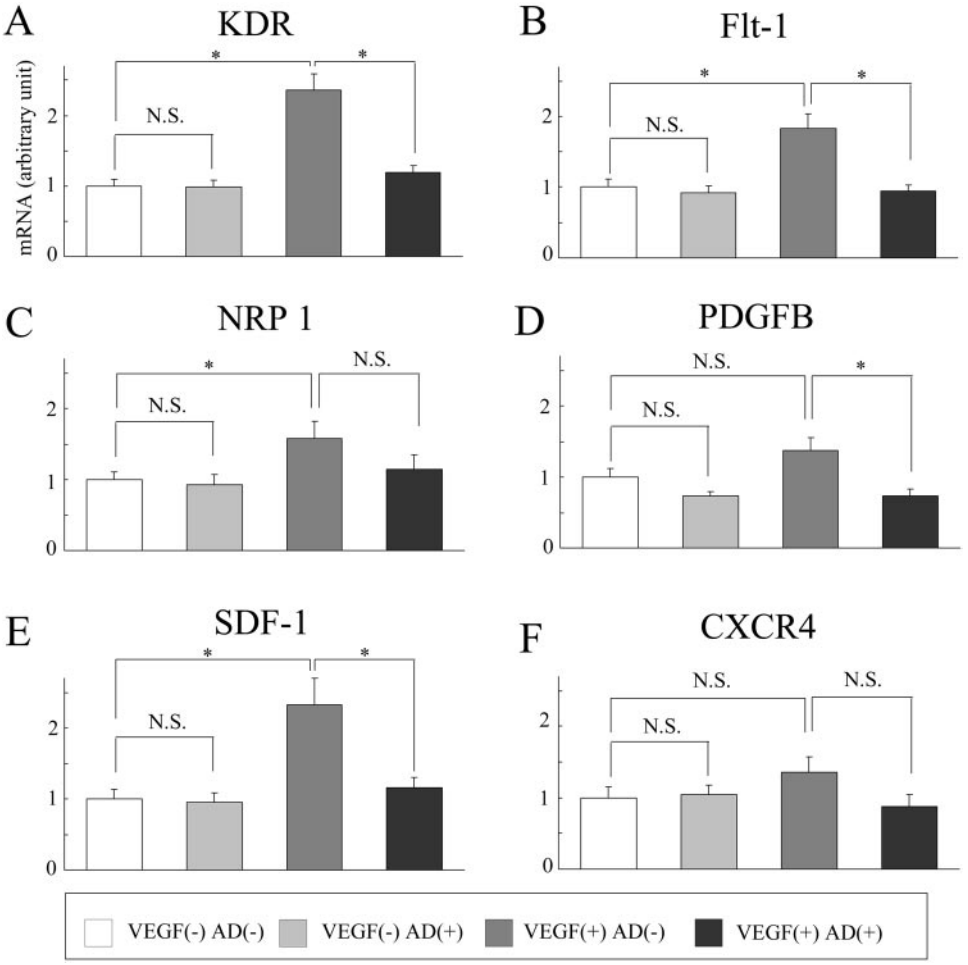

FIGURE 6. Effects of AD on mRNA levels of angiogenic molecules. After each treatment for 96 hours, mRNA of KDR (**A**), Flt-1(**B**), neuropilin-1 (**C**), PDGFB(D), SDF-1 (**E**), and CXCR4 (**F**) in retinal explants were quantified by real-time PCR. $n = 10$. $^*P < 0.05$.

amoeboid.[33] Our data suggest that AD partially inhibits the VEGF-induced motility of microglia.

AD Affects the Transcriptional Levels of Angiogenic Molecules

To evaluate the transcriptional level of angiogenic molecules, we treated retinal explants with VEGF, AD, or both for 96 hours. RNA was isolated and followed with reverse transcription and real-time PCR. We first evaluated three receptors of VEGF and found that VEGF treatment increased mRNA of KDR and Flt-1, which were significantly inhibited by AD without affecting changes in their basal levels (Figs. 6A, 6B). The transcriptional level of neuropilin-1, which is a coreceptor of VEGFR, was also increased by VEGF, but AD did not alter it either with or without VEGF treatment (Fig. 6C). Among these receptors, KDR induces significant stimulation in vascular endothelial cells and is expressed in tip cells abundantly.[23] The decrease in both tip cell motility and mRNA of KDR by AD treatment suggests that AD inhibits VEGF-induced activation of tip cells. We further analyzed the transcriptional levels of PDGFB, which is another tip cell marker,[23] and found that mRNA levels of PDGFB were also attenuated by AD treatment in the presence of VEGF (Fig. 6D), possibly providing further confirmation of tip cell insufficiency by AD treatment.

Recent studies have demonstrated that SDF-1 and its receptor, CXCR4, regulate angiogenesis.[26] The SDF-1/CXCR4 axis contributes to the motility of tip cells and microglia (unpublished data) and to the recruitment of EPCs to angiogenic sites.[5,34] Therefore, we also measured mRNA levels of these molecules and found that VEGF increased SDF-1 mRNA, which was suppressed by AD treatment (Fig. 6E). Compared with SDF-1, mRNA levels for CXCR4 did not alter with either VEGF or AD treatment (Fig. 6F). These data support the findings that tip cell motility and filopodial extension are attenuated by AD.

AD Attenuates Retinal Vascular Development in Neonatal Mice

We evaluated the effects of AD on in vivo retinal vascular development. Either AD (1 nmol/g body weight) or vehicle was intraperitoneally administrated on P2 neonatal mice, and retinas were harvested on P4. Immunostaining with PECAM demonstrated that AD significantly reduced the vascularized area of the developing retinas, compared with vehicle-treated ones (Figs. 7A–C, 7F). Interestingly, filopodia in tip cells were extended to avascular areas in vehicle-treated retinas, whereas AD treatment reduced the number of and distorted filopodia (Figs. 7D, 7E). These data are consistent with the data in retinal explants that AD at least partially inhibits retinal angiogenesis mediated by tip cell dysfunction.

DISCUSSION

We have analyzed the effects of a novel angiostatic cortisene, AD, on the multiple processes of retinal angiogenesis using time-lapse imaging. Previous publications showed that AA attenuates neovascular tufts in ROP models, whereas they could not determine whether AA suppresses the development or progression of the tufts.[10] In the present study, we demon-

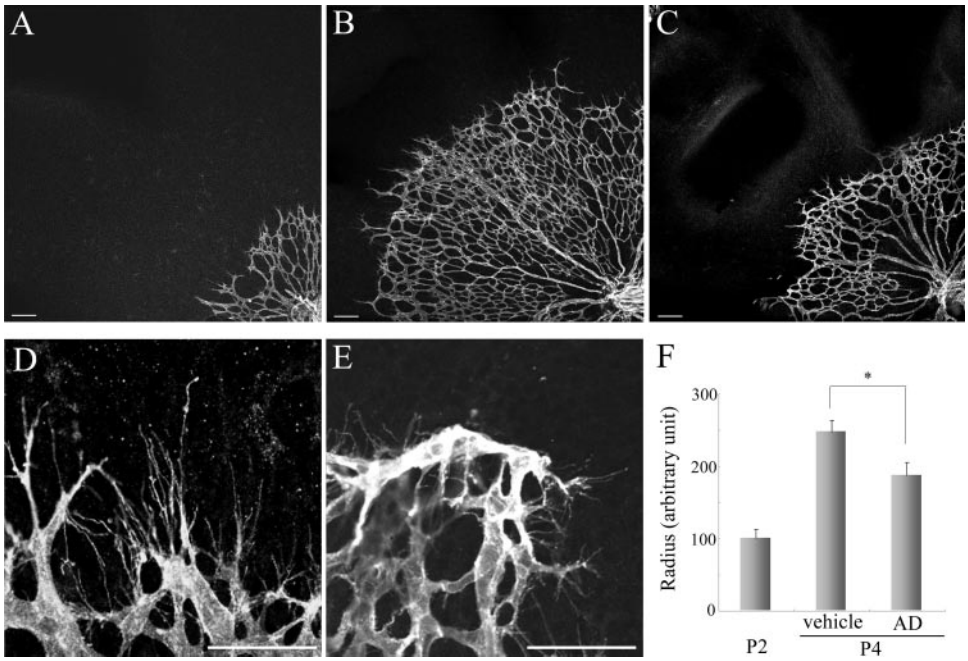

FIGURE 7. AD suppresses retinal vascular development. 1.0 nmol/g body weight AD was administrated intraperitoneally on P2 (**A**), and the vascularized area was analyzed by immunostaining with PECAM on P4. Compared with vehicle (**B**), AD reduced the vascularized area (**C**). Filopodial extension was also affected by AD (**E**) compared with the control (**D**). (**F**) The radius of the vascularized area was quantified. $n = 8$. $^*P < 0.05$. Scale bars: 100 μm (**A–C**); 50 μm (**D**, **E**).

strated that AD suppressed both the development and the progression of neovascular sprouts in retinal explants treated with VEGF. Further in vivo analysis in a model of retinal vascular development provided additional confirmation that AD suppressed the progression of sprouting angiogenesis with tip cell dysfunction.

When we consider the clinical application of antiangiogenic agents, it is helpful to fully understand whether they suppress the development or progression of sprouting angiogenesis. If drugs inhibit the development, we could expect preventive benefits against angiogenic diseases. On the other hand, when drugs attenuate the progression of angiogenesis or induce its regression, they are powerful therapeutic weapons against neovascularization that has already developed. This novel angiogenic system with time-lapse imaging may provide useful insights, and the results in the present study suggest that AD might be applied for both preventive and therapeutic purposes in VEGF-induced retinal angiogenesis.

Several antiangiogenic drugs are now being clinically assessed,[6,35–37] and combined therapies may even provide better therapeutic results in the near future. Further research regarding complementary molecular mechanisms may allow us to combine these drugs more effectively and with fewer adverse effects. In this study, we estimated the transcriptional levels of angiogenic molecules to investigate the molecular basis for the antiangiogenic activity of AD. mRNA levels for KDR and PDGFB, which are highly expressed in tip cells, were reduced by AD. This finding would be consistent with the decreased motility of tip cells in retinas treated with AD. In particular, the reduction of KDR mRNA might contribute to the disturbance of the elongation of sprouts and the decreased extension of leading edges because KDR is thought to serve as a receptor of attractive cues in tip cells.[23] Additionally, VEGF-induced KDR expression was reversed by AD treatment, without changes in basal KDR levels, suggesting that AD may inhibit signal transduction pathways activated by VEGF treatment in vascular endothelial cells. However, we must consider that KDR expression occurs in neuronal cells[38] and that other cellular sources of increased KDR remain to be elucidated.

We also analyzed mRNA levels for the SDF-1/CXCR4 axis because this axis regulates tip cell function and influences the expression levels of KDR and PDGFB (unpublished data). VEGF increased SDF1 mRNA, but not CXCR4, and AD reduced SDF-1 mRNA without changing basal levels. These results suggest that AD might negatively affect tip cell function by partial suppression of the SDF-1/CXCR4 axis. We have found that SDF-1/CXCR4 axis regulates the transcription of KDR and PDGFB in retinal vascular development (TM, unpublished data, 2009); decreased levels of KDR and PDGFB mRNA induced by AD treatment might also be regulated indirectly by SDF-1, though further pathway analysis remains to be performed.

VEGF-induced microglial motility was also suppressed by AD treatment. Because inflammatory cells, including macrophages, are often activated by Flt-1 signaling,[39] the observation that AD decreased VEGF-induced Flt-1 mRNA might be associated with the finding of decreased motility of microglia. AD also decreased VEGF-induced SDF-1 mRNA, which might contribute to the reduction in microglial movements because the SDF-1 receptor, CXCR4, is expressed in microglia.[40] In addition, we have observed that the inhibition of SDF-1/CXCR4 reduces microglial motility (unpublished data). Microglia contribute to the promotion of retinal angiogenesis,[31,32] and decreased Flt-1 and SDF-1 induced by AD may affect the reduction of neovascular sprouts after AD treatment. However, Flt-1 is also expressed in vascular endothelial cells[41] and has been shown to promote angiogenesis after ligand binding in endothelial cells.[42] Therefore, we must consider that Flt-1 reduction induced by AD treatment could have direct effects on endothelial cells and indirect effects mediated by microglia.

To evaluate the intervention effects on neovascular sprouts that had already developed, AD treatment was added after 96 hours of VEGF exposure. These experiments showed a definite reduction in the progression of neovascular sprouts. Most commonly, steroid derivatives have been shown to interact with their receptors, which then bind specific DNA sequences in the nucleus and regulate transcriptional levels, providing a so-called genomic effect.[43] Another rapid activity of typical steroid molecules, nongenomic effects, have recently been identified.[44,45] Several kinds of typical steroids activate signal transduction, including Src, MAPK, and Akt pathways, which are mediated at least partially by membrane steroid receptors; however, detailed mechanisms remain poorly defined.[46] Although AD was structurally derived from cortisol, permanent chemical modifications rendered the novel cortisene basically devoid of typical glucocorticoid activity. The specific AA or AD

receptor(s) responsible for angiostatic activities have not been isolated. In addition, the changes in transcriptional levels of angiogenic molecules discussed here could have been regulated by nongenomic effects and genomic effects.

In the present study, we demonstrated that AD suppresses both the development and the progression of neovascular sprouts, suggesting potential beneficial effects for preventive and therapeutic purposes in treating pathologic retinal angiogenesis.

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