The Effect of an Angiostatic Steroid on Neovascularization in a Rat Model of Retinopathy of Prematurity

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PURPOSE. The inhibition of angiogenesis by angiostatic steroids has been demonstrated in a variety of systems, including rabbit and rat cornea. There is considerable interest in the therapeutic potential of this class of compounds for angiogenic ocular conditions such as diabetic retinopathy, macular degeneration, and retinopathy of prematurity (ROP). This study was designed to test the capacity of an angiostatic steroid, anecortave acetate, to inhibit retinal neovascularization using a rat model of ROP and to investigate the mechanism of the effect.

METHODS. At birth, rats were placed in an atmosphere of varying oxygen that produces retinal neovascular changes that approximate human ROP. The rats then received intravitreal injections of either anecortave acetate or vehicle at varying times, and all were subsequently placed in room air. Retinas were assessed for plasminogen activator inhibitor (PAI)-1 mRNA level by RNase protection assay at 1, 2, and 3 days after injection and for normal and abnormal blood vessel growth 3 days later.

RESULTS. A significant reduction in the severity of abnormal retinal neovascularization was observed in the steroid-treated eyes compared with vehicle-injected eyes in ROP rats, yet the extent of normal total retinal vascular area was not significantly different. The drug had no effect on either retinal vascular area or neovascularization when tested in room air–raised control rats. Drug-injected eyes demonstrated a six- to ninefold increase in PAI-1 mRNA at 1 to 3 days after injection.

CONCLUSIONS. This study represents the first therapeutic effect of an angiostatic steroid in an animal model of neovascular retinopathy. Additionally, the induction of PAI-1 indicates a mechanism of action for this class of compounds, and this is a novel finding in vivo. Because anecortave acetate significantly inhibited retinal angiogenesis in this model, while not significantly affecting normal intraretinal vessels, it holds therapeutic potential for a number of human ocular conditions in which angiogenesis plays a critical pathologic role. (Invest Ophthalmol Vis Sci. 2001;42:283–290)}
appear in a retinal pattern mimicking that occurring in premature infants with ROP.

To our knowledge, this report is the first demonstration of a therapeutic influence of an angiostatic steroid on retinal angiogenesis. In addition, we have initiated investigations into the mechanism of the angiostatic influence of anecortave acetate. We have focused on the ability of the compound to induce plasminogen activator inhibitor (PAI)-1 mRNA. This endogenous protease inhibitor counterbalances the action of plasminogen activator—an upstream initiator of proteolysis of the extracellular matrix. The induction of PAI-1 by angiostatic steroids has been demonstrated in vitro, but to date this influence has not been identified in animal studies.

**METHODS**

**Oxygen Exposure Protocol**

Within 4 hours after birth, randomized litters of Sprague-Dawley albino rats were placed with their mothers in an Isolite infant incubator, in either room air (RA) or variable oxygen environments (VOE). In the case of VOE rats, the oxygen concentration in the incubator was adjusted to alternate between 50% and 10% every 24 hours for 14 days, after which time all rats were removed to room air. Oxygen concentrations were chosen to produce bloodstream oxygen tensions (\( \text{PaO}_2 \)) in rats similar to those of premature infants in whom ROP develops as determined by extensive blood gas analysis of premature infants in the NICU of Arkansas Children’s Hospital. All other conditions (e.g., light exposure, temperature, feeding, etc.) were similar for VOE and RA treatment groups. These experiments were approved by the Vanderbilt University School of Medicine Animal Care and Use Committee and the ARVO statement for the use of animals in ophthalmic and vision research.

**Intravitreal Injections**

Rats were anesthetized with a 40 mg/kg intraperitoneal injection of ketamine along with topical application of 0.5% proparacaine to the eye. The fused eyelids were opened and temporally canthotomized, when necessary, and then local anesthetic was reapplied, followed by 1% mydriacyl. While proptosing with gentle pressure on the lids, the point of entry. Noninjected eyes also were treated with topical proparacaine and antibiotic to control for the potential of these agents to influence retinal vessel growth. Vitreous hemorrhage occurred immediately after injection in two cases, and these rats were eliminated from the study and replaced with alternates.

**Drug Treatment Protocol**

Aneecortave acetate [4,9(11)-Pregnadien-17\(^{\alpha,21}-dihol-3,20-dione-21-acetate] (Fig. 1) was obtained from Alcon Laboratories, Inc. (Fort Worth, TX). The experimental protocol for these studies is presented in Figure 2. At 14 days, immediately after removal of the VOE rats from the exposure chamber, rats in both the RA and the VOE groups received an intravitreal injection in the left eye. Half of the rats \((n = 12)\) received 5 \(\mu\)l of a 10% suspension of anecortave acetate and the remainder \((n = 12)\) received 5 \(\mu\)l of vehicle \((70:20:10 \text{ by volume, polyethylene glycol: phosphate buffered saline: EtOH})\). After 2 days in room air (Day 16), the injection protocol was replicated in the right eye of each animal (i.e., same agent and volume as in the left eye). The dosage of steroid used was based on dose/response optimization trials previously performed in our laboratory (data not shown).

Multiple control groups were added to control for an effect of intravitreal injection of drug or vehicle on the contralateral eye. At 14 days of age, additional VOE rats were given drug \((n = 6)\) or vehicle \((n = 6)\) injections in one eye only; the contralateral eye remained uninjected. Still other VOE animals \((n = 10)\) received no injection in either eye, and data from both eyes of these rats were averaged. All rats in these experimental and control groups were killed by decapitation under deep anesthesia at 18 days of age, and their retinas were dissected for analyses of normal retinal vascular development and abnormal retinal angiogenesis (i.e., retinal neovascularization). There was no attrition of animals during the experiments, but the retina of one eye injected with vehicle at 16 days was lost to dissection artifact.
and the retina of one un.injected eye was lost to processing artifact. All other eyes yielded data.

To determine the effect of anecortave acetate on the normal development of intraretinal blood vessels, a limited trial was conducted in rats raised in room air. Seven-day-old rats were chosen for this experiment because the growth of retinal blood vessels from the optic nerve head, where the process starts, to the far retinal periphery where it ends, is about half-complete at this age. Any inhibition of the process over the next several days is easily measured. At 7 days of age, 5 μl of 10% anecortave acetate was injected into the left eye and 5 μl of vehicle into the right (n = 8). Additional 7-day-olds were left with no injection (n = 5). These rats were killed at 10 days of age, a day or two before the time when the first retinal vessels normally reach the ora serrata.

**Effect on Retinal Vascular Development**

After enucleation and dissection of the retinas in 10% neutral buffered formalin, the retinas were flattened and stored overnight at 4°C. Retinas were then processed for adenosine diphosphatase (ADPase) histochemistry using a modification of the method of McLeod and coworkers. This process primarily stains vascular endothelial and their stem cells in rats of this age. Images of ADPase-stained retinas were digitized and captured (Imagegrabber 2.0, Neotechnix, Hampshire, UK). Retinal areas containing blood vessels were then traced on the computer monitor face with an interactive stylus pen (FT Data Systems, Stanton, CA). The operator was masked with respect to the treatment. The area within the trace was calculated with image analysis software (Enhance 3.0, Microfrontier, Des Moines, IA) and is reported in mm². Measurements of this parameter were recorded and statistically significant differences between the treatment groups were determined by analysis of variance with Scheffe’s post hoc procedure. Data normality was determined by the D’Agostino test. Statistical analysis of the data from rats killed at both 10 and 18 days of age was conducted in this manner.

**Effect on Retinal Neovascularization**

To determine the effect of anecortave acetate on retinal neovascularization, the degree of vascular pathology was assessed in the flattened, ADPase-stained retinas. As the retinas were flattened, care was taken to ensure that each of the four quadrants was nearly equal in size. Each of these quadrants was divided into three theoretical clock hours. Each clock hour that was occupied by neovascular growth was counted, yielding a semiquantified measure of severity with values ranging from 0 (no pathology) to 12 (most severe). In this context, neovascular growth included any preretalinal vascular growth and any regions of dense arterial budding within the plane of the superficial vessel plexus (see Fig. 6, bottom center panel, open arrow and black arrow). Neither of these two features is observed during normal vessel development in room air-raised rats, and each is seen in human ROP. Determinations were confirmed at 400× magnification. Masked assessments were conducted by three independent observers; the median of these individual assessments is reported. The clock hour data from the present study were not normally distributed, as determined by the D’Agostino test. Therefore, significant differences were determined by the Kruskal–Wallis test with a Scheffe’s post hoc analysis.

Following these neovascular assays, several (n = 5) of the flattened retinas were selected and processed for histologic sectioning. Briefly, the retinas were removed from the microscope slide, fixed in 2.5% glutaraldehyde, dehydrated in an ethanol series, and infiltrated and embedded in Embed 812 (Polyesciences, Warrington, PA). Sections of 0.5 μm thickness were stained with 1.0% toluidine blue (see Fig. 5).

**RNase Protection Assay**

Several experiments were performed to elucidate the mechanism of action of anecortave acetate in the retinal tissue. Twelve rats each from three separate variable oxygen experiments (n = 36) were removed from the exposure chamber, anesthetized, and intravitreally injected with anecortave acetate, vehicle, or nothing. They were then killed 1, 2, or 3 days later. Retinas were dissected, three retinas from each treatment were pooled, and the samples were flash frozen in liquid nitrogen. Duplicate experiments were also conducted using six room air-raised rats similarly injected at 14 days and killed one day later. PolyA⁺ RNA was isolated from frozen retinas using the micro mRNA isolation kit (Amersham Pharmacia Biotech, Piscataway, NJ). The RNA integrity was confirmed by Northern blot analysis of the samples with Psoralen biotin-labeled β-actin antisense RNA probe (Ambion, Austin, TX).

Antisense RNA probe was generated from the 3’ end of rat PAI-1 cDNA (a generous gift from T. D. Gelehrter, Department of Human Genetics, University of Michigan Medical School, Ann Arbor, MI). The SK(−) plasmid containing the PAI-1 cDNA was linearized by restriction digestion with HindIII. A 445 nucleotide-long 58UTP labeled cRNA was transcribed from 1 μg of the linearized template using T3 RNA polymerase (Ambion). A 210 bp nucleotide β-actin antisense probe also was transcribed from linearized pTRIPLeScript vector (Ambion) using T3 RNA polymerase and was used as an internal standard for RNA levels. A 50-fold reduction in the specific activity of the β-actin probe was achieved by the addition of cold UTP. This was necessary to increase the sensitivity of the assay, owing to the excess levels of β-actin relative to PAI-1 mRNA in cells. The riboprobes were size-fractionated on 5% denaturing polyacrylamide gels and full length probes were eluted out of gel slices.

Coprecipitation of 2–8 × 10⁴ cpm each of PAI-1 and β-actin riboprobes (gel-purified) was performed with 0.3 to 0.6 μg individual PolyA⁺ samples using 50 μg tRNA as a carrier. The RNA/riboprobe coprecipitations were reabsorbed in Hyb-speed hybridization buffer (Ambion). Immediately after denaturation at 95°C for 4 minutes, samples were hybridized at 68°C for at least 10 minutes and digested at 57°C for 30 minutes, with 10% volume of an RNase cocktail (RNaseT1/RNaseA at 1:2 vol:vol ratio) diluted at 1:100 in digestion buffer. The RNases were inactivated and the protected probe (PAI-1 and β-actin) fragments were precipitated by the addition of 2X volume of an inactivation/precipitation buffer (Ambion). The precipitates were re-suspended in formamide loading buffer and separated on a 5% denaturing polyacrylamide gel containing 7 m/l urea. In vitro transcribed, 58UTP labeled 100 to 500 bp fragments generated from a Century marker template were used as molecular weight markers. Gels were transferred to 3-mm paper, wrapped in plastic wrap and exposed to X-ray film with intensifying screen at -80°C from overnight to a week to obtain desired signal intensity for autoradiography.

The PAI-1 probe (~445 nt) is complementary to only 376 nt of the PAI-1 mRNA sequence and is expected to produce a protected fragment of that length. The β-actin probe (210 nt) is expected to produce a 129 nt-protected fragment when hybridized to rat mRNA. Each treatment RNA sample was extracted using retinas pooled from three pups.

In the presence of excess probe, the intensity of the protected bands is directly proportional to the relative levels of the message. With the housekeeping β-actin gene serving as an internal control, the PAI/β-actin pixel density ratio reflects the differences in PAI-1 mRNA levels between treatment groups.

**RESULTS**

Anecortave acetate had no significant effect on the extent of normal retinal vascular development. An intravitreal injection of the steroid in normal 7-day-old rats produced no significant differences in retinal vascular area, compared with retinas from noninjected or vehicle-injected eyes. At 10 days of age, retinal vascular area was 30.1 ± 2.8 mm² in vehicle-injected eyes and 28.4 ± 2.4 mm² in anecortave acetate–injected eyes (P = 0.25). This corresponded to 92% and 87% of the total retinal area in the two groups, respectively.

Analysis of rats raised in the VO environment also demonstrated no significant effect of the steroid on retinal vascular...
Retinas that were injected at day 16 yielded vascular areas of 0.55 mm² for vehicle in retinas injected at day 14 (P = 0.07). There was no significant difference noted for rats injected on Day 14 versus Day 16 (P = 0.0001). On Day 16 results were P = 0.03 when compared to vehicle and P < 0.001 when compared to drug-injected eyes.

There was no apparent effect of vehicle or anecortave acetate on the retinal vascular area of contralateral eyes. Rats receiving vehicle injections in one eye exhibited vascular areas in contralateral, un.injected eyes that were not different from those of rats receiving no injection in either eye. Rats receiving anecortave acetate injections in one eye exhibited vascular areas in contralateral, vehicle-injected eyes that were not different from those of vehicle injected eyes of rats with no injection in contralateral eyes.

To assess retinal neovascularization, we used a semiquantitative methodology involving a clock-face scale of neovascular growth severity (see Methods). This method was chosen because, although not rigorously quantitative, it has a clear corollary in the clinical classification of ROP and is widely accepted by laboratories that employ ROP animal models. Furthermore, multiple transverse sections are impractical and, because of the asymmetry of the pathology, are prone to sampling errors. Finally, when these sampling errors were controlled by serially sectioning entire eyes (700–900 sections/eye) in a similar rat model, the number of preretinal nuclei was highly correlated (r² = 0.95, P < 0.0001) with clock-hour measures.

There was significantly less retinal neovascularization in anecortave acetate–injected VOE rats than in vehicle-injected VOE rats, regardless of injection time (Fig. 3B). Median values were 2 clock hours, ranging from 0 to 4 (mean ± SD, 2.1 ± 1.2 clock hours), for anecortave acetate vs. 6 clock hours, ranging from 3 to 8 (mean ± SD, 5.8 ± 1.6 clock hours) for vehicle at the first injection time (P < 0.0001). Median values resulting from the second injection were 3 clock hours, ranging from 1 to 5 (mean ± SD, 3.9 ± 1.1 clock hours), for anecortave acetate vs. 6 clock hours ranging from 3 to 10 (mean ± SD, 5.9 ± 1.6 clock hours), for vehicle at the second injection time (P < 0.0002). Among drug-treated eyes, there was no significant difference in the severity of neovascularization between treatment times (P = 0.21).

Noninjected eyes of oxygen-exposed rats showed significantly more pathologic neovascularization (median and range: 8, 5 to 12; mean ± SD, 8.2 ± 1.7 clock hours) than either vehicle- (median and range: 6, 3 to 10; mean ± SD, 5.8 ± 1.6, P < 0.0001) or drug-injected (median and range: 2.5, 0 to 5; mean ± SD, 2.5 ± 1.3, P < 0.0001) eyes, when data from both injection times are combined.

TABLE 1. Confidence Intervals for Combined and Paired Comparisons of Neovascularization Measures

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A, anecortave acetate.
No room air–raised rat demonstrated retinal neovascularization. Control groups demonstrated no effect of vehicle or drug on neovascularization in contralateral eyes. Table 1 lists the critical values and confidence intervals for combined and paired comparisons of neovascular retinal clock hours.

A comparison of two retinas from 18-day-old rats treated at the time of removal from the exposure chamber (Day 14) is shown in Figure 4. The arrows designate the location of pre-retinal tufts of vessels. The presence of these vessels in a focal plane above the inner limiting membrane was confirmed at high magnification. Figure 5 shows an example of the retinal pathology caused by our 50%/10% alternating oxygen exposure (VOE) in an eye that received no intravitreal injection. This figure serves to illustrate several characteristics of the pathology, which are detailed in the legend.

Analysis of plasminogen activator inhibitor-1 mRNA levels provided a clue as to the mechanism of action of anecortave acetate. Retinal PAI-1 mRNA was not detectable by our system in non-injected eyes (Fig. 6). Vehicle injection caused an increase in PAI-1 mRNA to detectable levels at 1 day after injection in both room air- and oxygen-treated rats. The signal dropped below the level of detection at later timepoints in vehicle-treated eyes. The retinal levels of PAI-1 mRNA in

**Figure 4.** Antiangiogenic effect of anecortave acetate. Two retinas are compared, one from a vehicle-injected eye (right) and the other from an eye injected with AA (left). These retinas are representative samples and illustrate the difference in retinal neovascularization between the two groups. The number and size of pre-retinal tufts in the vehicle-injected eye are greater than that of the drug-treated eye; these are the hallmarks of retinopathy.

**Figure 5.** Histopathology of retinal neovascularization. The top photograph depicts a transverse section through an area of dense tufts of preretinal vessels. On the lower left is a photograph of the same retina in a flat preparation. The inferior quadrant of this ADPase-stained retina (bottom, middle panel) serves to illustrate several characteristics of the pathology, including the avascular (a) peripheral retinal region (not included in assessment of neovascularization), abnormal budding of arterioles at the peripheral avascular/vascular interface (black arrow), sheets of preretinal vessels associated with peripheral venules (open arrow), and a subretinal hemorrhage (white arrow in the upper left of the panel; not included in the assessment of neovascularization) beneath a large preretinal tuft. On the lower right is a higher magnification photograph of an area of the transverse retinal section, illustrating a tuft of preretinal neovascularization. These tufts contain little blood volume because they lack an extensive network of lumenized capillaries. Hence surface marker staining techniques (such as ADPase staining by tissue submersion) are superior to infusants or perfusants (such as fluorescein and ink) in highlighting neovascular regions.
accumulating evidence argues for the suggestion that angiostatic steroids inhibit the dissolution of basement membrane and other extracellular matrix components.

In 1967, Pandolfi described a restructuring of extracellular matrix that occurred at actively extending vessel tips and that involved secretion of a protease known as plasminogen activator. It is now known that two distinct classes of plasminogen activators exist: a urokinase-type (u-PA), which participates in extracellular matrix breakdown during endothelial cell migration, and a tissue-type (t-PA), important in thrombolysis. Established vessels secrete only the t-PA form, but studies using a guinea pig corneal neovascularization model demonstrated that endothelial cells in new vessel sprouts secrete u-PA exclusively.

Studies by Ashino-Fuse and colleagues explored the mechanism by which novel angiostatic steroids, already in use clinically for mammary carcinomas, exerted their effect. These workers concluded that angiostatic steroids suppressed PA activity, either by inhibiting production of the enzyme itself at the transcription or translation stage, or by inhibition of its secretion. The authors did not exclude the possibility of an increase in the level of an endogenous inhibitor of PA.

More recently, it has been demonstrated that angiostatic steroids exert their inhibitory effect on endothelial cell growth in vitro by increasing the synthesis of PAI-1. This induction of PAI-1 then inhibits u-PA activity, which is essential for the invasive aspect of angiogenesis — the breakdown of vascular endothelium basement membrane and extracellular matrix. Therefore, the result of steroid-induced suppression of PA function is that endothelial cells cannot proliferate and migrate toward an angiogenic stimulus to participate in new blood vessel formation. Our results provide the first evidence that angiostatic steroids may operate by the same mechanism in vivo. The anecortave acetate–mediated induction of PAI-1 mRNA is rapid (by 24 hours), is sustained (at least 3 days) and is robust (since constitutive levels of PAI-1 mRNA are undetectable by band densitometry in our system). The effect of this PAI-1 mRNA induction on downstream protease activity is currently under investigation.

Aneccortave acetate is designed to be devoid of conventional hormonal activity. It contains the important structural modification of a 9-11 double bond that replaces the 11β-hydroxyl group, which is essential for glucocorticoid and mineralocorticoid activities. Aneccortave acetate demonstrates no significant glucocorticoid-mediated anti-inflammatory agonist activity in vitro or in vivo inflammation assays, including carrageenan-induced footpad edema in rats, endotoxin-induced uveitis in rabbits, and IL-1 induction in cultured human U937 cells. In addition, anecortave acetate does not block the anti-inflammatory activity of dexamethasone, so it is also devoid of glucocorticoid antagonist activity. The absence of glucocorticoid activity is important because of the significant ocular side effects associated with ocular glucocorticoid therapy.

Trials of the angiostatic capacity of anecortave acetate have been conducted in chick chorioallantoic membrane, in a rabbit corneal neovascularization model, where 90% inhibition of the area of new corneal blood vessels resulted; and in an intraocular tumor model, where net tumor weight was held to less than 1/3 that of control. Tumor inhibition was concluded to be the result of the angiostatic properties of anecortave acetate, since neither the parent compound nor its deacetylated metabolite affected tumor cell proliferation in vitro. Notably, in each of the latter two studies the mode of administration was topical ocular application. Aneccortave acetate has the additional attributes that it is relatively nontoxic (no studies describing its use have reported attrition), and its bioactivity is apparently independent of species or cause of the angiogenesis, making its therapeutic value more promising.
The present study demonstrates a phenomenon that our laboratory has observed over the past several years while delivering angiogenic agents intravitreally — namely, the therapeutic effect of vehicle injection. Preliminary studies have yielded an identical effect from dry needle puncture. In the present study, vehicle injections caused the reduction of retinal vascular area by approximately 15% and, more importantly, abnormal angiogenesis by approximately 30% when data from both injection times are combined. We postulate that the release of endogenous factors from the wound site and/or the surrounding retinal area may play a role in this effect, suggesting that other potential therapeutic agents for proliferative retinal disease might be constituents of the retina's endogenous battery of cytokines. Based on this hypothesis, studies are underway to determine what endogenous retinal cytokines hold the capacity for angiogenesis and which one(s) are responsible for the effect of vehicle injection. In fact, the effect may be partly PAI-1-mediated, as indicated by the retinal response to vehicle injection illustrated in the pixel density bar graph (6B). Unfortunately, the single assay most representative of the effect of AA on PAI-1 induction in oxygen-treated rats (6A) did not show this vehicle effect.

ROP is a condition of growing concern in the United States. The incidence of blindness associated with this condition can be extrapolated from previous estimates to nearly 700 infants per year. Some permanent vision loss can be expected in nearly 4000 infants annually due to ROP. Among pathologic ocular conditions, ROP has the unique feature that normal and abnormal vessel growth occur simultaneously in very close proximity. The inhibitory effect of angiostatic steroids on normal retinal vascular development must be considered carefully before these, or like agents, can be developed for therapeutic application to ROP.

The limited effect of anecortave acetate on normal vessel development (~15% inhibition versus vehicle at either injection time), while it profoundly affected pathologic neovascularization (50% or greater inhibition versus vehicle, depending on injection time), is compelling. At least two possibilities exist for this discrimination: 1) Early intraretinal vessel development depends largely on differentiation of mesenchymal precursors (vasculeogenesis) rather than mitosis and budding of existing vessels (angiogenesis), which produces the neovascularization of ROP. It may be that basement membrane and extracellular matrix remodeling, a known requirement of angiogenesis, is less important to, or not required of, retinal vasculegenesis. Thus inhibition of the proteases that perform this remodeling function might preferentially target preretinal vessels that grow by an angiogenic process. 2) Preretinal vessels may have been available to the drug because of their immediate contact with the vitreous, yet intraretinal vessels were not available. The ocular pharmacokinetics of angiostatic steroids are only partly defined, but daily examination of the drug-injected eyes in this study would indicate that the highly hydrophobic anecortave acetate was not rapidly cleared from the vitreous. In either case, one must evaluate these drugs on the backdrop of the invasiveness and limited favorable outcome of cryo- or laser therapy — the currently accepted methods of clinical intervention for ROP. When viewed in this light, anecortave acetate or similar molecules may offer rational alternatives to the current methods of surgical intervention. Furthermore, in other retinopathies, where the retinal environment is not complicated by normally developing vessels, this agent may inhibit abnormal retinal angiogenesis while not effecting normal, mature blood vessels in the same region.

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References


