

# Differential retinal angiogenic response to sustained intravitreal release of VEGF and bFGF in different pigmented rabbit breeds

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## Abstract

**Purpose.** To determine if two different breeds of pigmented rabbits can demonstrate differences in the degree of inducible angiogenesis within the retina.

**Methods.** Non-biodegradable Hydron pellets approximately 1.5 mm in diameter containing both vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) were implanted intravitreally over the optic disk of either Dutch belt rabbits or New Zealand White/Black satin cross rabbits. Control animals from both groups were implanted with blank Hydron pellets. Animals were examined periodically over a 30-day period following implantation. Results were documented by fundus photography and fluorescein angiography. Stages of neovascularization (NV) were graded between +1 (proliferative) and +4 (total NV) with +5 for NV complicated by hemorrhage and/or retinal detachment.

**Results.** The angiogenic response in the retinas of pigmented NZW/Black satin cross rabbits (N = 5) following implantation of VEGF/bFGF-containing pellets varied extensively from the Dutch belt animals (N = 7). In the Dutch belt rabbits, grading of the angiogenic response demonstrated either +4 or +5 between day 20 and day 30 after implantation. In contrast, the NZW/Black satin cross animals gave a more muted response with a maximum grade of +2 following exposure to the same amount of VEGF and bFGF. Control eyes that received only blank pellets showed no evidence of retinal NV in either the Dutch belts (N = 5) or the NZW/Black satin cross rabbits (N = 5). Statistical analysis showed a significant interaction effect for breed and pellet

type (F = 44.85 with 1 df, p < 0.00005), indicating a difference between the breeds in the angiogenic response to the pellet. Moreover, both the NZW/BSC and Dutch belt rabbits displayed a significant increase in angiogenesis with the VEGF/bFGF pellet in comparison to the blank pellet (p = 0.037 and p < 0.00005, respectively).

**Conclusions.** These studies indicate that two different breeds of pigmented rabbits exhibit different angiogenic responses to the same amount of both VEGF and bFGF. Florid retinal NV leading to hemorrhage, fibrovascular membrane formation, and traction retinal detachment occurred in the Dutch belt rabbits while tortuosity and dilatation of existing blood vessels with subsequent regression occurred in the NZW/Black satin cross animals. Such differences in the angiogenic response may be due to differences in the genetic background of these animals. If genetic heterogeneity exists for angiogenic responses, then understanding the genetic role in the regulation of angiogenesis will lead to the design of more effective anti-angiogenic agents and can provide predictive outcomes of individual responses to therapy.

**Keywords:** animal model; basic fibroblast growth factor; hemorrhage; retinal angiogenic differences; vascular endothelial growth factor

## Introduction

Posterior segment angiogenesis is the final common pathway leading to visual loss in retinopathy of prematurity (ROP),

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diabetic retinopathy, and choroidal disorders.<sup>1-3</sup> Diabetic retinopathy is the major cause of new blindness in the working age group between the ages of 18 and 65 years of age while ROP is the leading cause of visual loss in newborns.<sup>2,4</sup> Another posterior segment neovascular process is subretinal neovascularization (SRN), which is the determinant of the disciform process that is responsible for the majority of visual loss in age-related macular degeneration (AMD).<sup>5,6</sup> In developed countries with people older than 50 years of age, AMD is a common cause of legal blindness.<sup>7</sup>

A florid model of retinal neovascularization (NV) in the relatively avascular rabbit retina is produced through simultaneous sustained intravitreal release of both vascular endothelial factor (VEGF) and basic fibroblast growth factor (bFGF), which is a non-specific fibroblast growth factor that acts on numerous cell types<sup>8</sup> and apparently provides angiogenic synergism with VEGF.<sup>9</sup> Following rapid onset of VEGF/bFGF-induced retinal NV, significant intravitreal hemorrhaging and subsequent traction retinal detachment occurs rapidly and reproducibly over a period of 3 weeks. A smaller subset of animals presenting with a lesser degree of hemorrhaging had repeated cycles of hemorrhage with spontaneous absorption and subsequent formation of whitish fibrovascular membranes. Other animal studies have reported that a mild reversible tortuosity and dilatation of existing rabbit blood vessels in albino rabbits occurred after sustained release of VEGF alone that eventually regressed<sup>10</sup> and that repeated intravitreal injections of VEGF in an adult primate produced retinal ischemia and microangiopathy.<sup>11</sup>

Interestingly, the hallmark of proliferative diabetic retinopathy involves initially extensive active proliferation of new blood vessels with visual loss resulting from vitreous hemorrhage or fluid exudation of the leaky fragile vessels.<sup>12,13</sup> Eventually with time, the proliferating vessels become fibrotic, involute, and produce retinal traction leading to complications such as retinal detachment. Finally, the disease becomes inactive with no further loss of vision.<sup>14</sup> These clinical manifestations of neovascular retinopathies also appear in the rabbit retina following sustained simultaneous intravitreal exposure to VEGF and bFGF and thus confirm the critical role of vascular-specific growth factors in retinal neovascular diseases.<sup>15-17</sup> Since the rabbit retina does not possess a macula, neither macular edema nor macular ischemia from capillary dropout of the original retinal vessels can be ascertained in this rabbit model of retinal NV. However, clinically significant retinal edema from leaky retinal blood vessels can be monitored by non-invasive imaging technologies.<sup>18-20</sup>

Differences in the ability of an individual to grow new blood vessels may influence the rate of progression of these posterior segment angiogenic-related diseases that lead to severe visual loss. Current characterization of genes responsible for angiogenic phenotypic heterogeneity and tissue specificity<sup>21,22</sup> eventually will provide increased understanding of genetic factors involved in pathologic angiogenic dis-

orders. The existence of genetic heterogeneity of angiogenesis has been shown in mice<sup>23</sup> where different strains of inbred mice have an approximately 10-fold range of response to growth factor-stimulated angiogenesis in the corneal micropocket assay along with a differential sensitivity to angiogenesis inhibitors between strains.

Moreover, some diabetic patients with no known predisposing risk factors develop severe retinopathy while others do not progress to retinopathy. Since 20% of the diabetic population does not develop significant retinopathy, genetic risk factors have been proposed to explain this observation.<sup>24</sup> Studies on such putative genetic factors have suggested that certain HLA haplotypes are associated with a greater incidence of diabetic retinopathy.<sup>25,26</sup> In addition, the progression of the late form of AMD with choroidal NV is rare for African-Americans in comparison to Caucasians.<sup>27</sup> Whether degree of pigmentation is a reflection of angiogenic heterogeneity is unclear; and whether variations of angiogenic responses occur within different strains or breeds of pigmented animals also is poorly understood. Therefore, the aim of this study is to determine if two different breeds of pigmented rabbits with highly pigmented retinal pigment epithelium (RPE) have different retinal neovascular responses to sustained intravitreal release of VEGF and bFGF.

## Materials and methods

### Intravitreal implantation of sustained-release pellets

Animals were treated according to the tenets of the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research and to the Declaration of Helsinki and The Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH 80-23). Both adult male Dutch belt rabbits and New Zealand white/Black satin cross (NZW/BSC) rabbits weighing between 2.0 and 3.0 kg were anesthetized with 35 mg/kg intramuscular injection of ketamine hydrochloride (Phoenix Pharmaceuticals, Inc., St. Joseph, MO, U.S.A.) and 5 mg/kg xylazine (Lloyd Laboratories, Shenandoah, Iowa, U.S.A.), respectively. Pupils were dilated with 2.5% phenylephrine (Alcon, Fort Worth, TX, U.S.A.) and 1% tropicamide (Baush & Lomb, Tampa, FL, U.S.A.).

Sterile preparations of both human recombinant VEGF 165 and recombinant bFGF (Pepro Tech, Rocky Hill, NJ, U.S.A.) were incorporated into a Hydron NCC polymer (Hydromed Sciences, Cranbury, NJ, U.S.A.) following specifications of the manufacturer. Also, solutions that contained PBS alone were incorporated into the Hydron polymer to produce pellets that acted as negative controls. Intravitreal implantation of the sustained-release polymeric pellets containing both growth factors bFGF and VEGF was performed over the optic streak of the NZW/BSC rabbits (N = 5) and Dutch belt animals (N = 7). Moreover, 20 µg bFGF-containing pellets were implanted intravitreally (N = 2) in addition to 20 µg VEGF (N = 2) in the Dutch belt rabbits.

Negative control animals received blank polymeric pellets containing only PBS for both Dutch belts (N = 5) and NZW/BSC (N = 5). Although the release rates of VEGF and bFGF in these polymeric pellets have been determined under *in vitro* conditions in phosphate-buffered saline over a 1-week period,<sup>28</sup> such data may not reflect actual release rates under *in vivo* conditions within the formed vitreous of the rabbit.<sup>29,30</sup>

Briefly, the conjunctiva was opened under a Zeiss operating microscope; and a 2 mm incision was made in the sclera approximately 2 mm posterior to the limbus. A second minor sclerotomy was performed for insertion of a retinal pick; alternatively, a syringe with a 30-gauge ½ inch needle was utilized. The pellet was grasped with an intraocular forcep, inserted through the sclerotomy into the vitreous cavity, and positioned in the space over the optic disk using either a retinal pick or a 30-gauge needle to maintain positioning of the pellet as the forcep was removed.<sup>9</sup> The first sclerotomy then was closed with 8-0 vicryl suture. Finally, 0.3% ciprofloxacin drops (Alcon, Fort Worth, TX, U.S.A.) were applied to the ocular surface following conjunctival closure.

#### Clinical evaluation of animals by ophthalmoscopy, and fundus photography

After anesthesia and dilation, eyes were examined by indirect ophthalmoscopy at baseline and after surgery at 24 hrs, 48 hrs, 4 days, 7 days, 14 days, 21 days, and 30 days. Results were documented by fundus color photography utilizing a portable Kowa Genesis retinal camera. In two rabbits implanted with VEGF/bFGF-containing pellets and in one rabbit implanted with a blank pellet, FA was carried out between 7 and 8 days after pellet implantation. At 30 days after intravitreal pellet implantation, animals were anesthetized as described previously and were sacrificed immediately by an intravenous overdose of pentobarbital.

#### Grading of retinal NV

A photographic grading system of retinal NV in this rabbit model has been described previously.<sup>9</sup> Specifically, fundus photographs were arranged in a temporal sequence for evaluation on the progression of the new blood vessels over time. The photographs were evaluated in a masked manner and scored by using four grades. Grade 0 displayed no vascular abnormalities in either the optic disk or the vascularized medullary rays. Grade +1 showed marked dilation and engorged tortuosity of the existing blood vessels in both the optic disk and medullary rays. Grade +2 displayed microvascular abnormalities, which presumably reflect new capillary buds although light and electro-microscopic studies have not been completed for confirmation. Grade +3 showed highly identifiable individual capillary loops growing into strands involving the optic disk and parts of the medullary rays. Grade +4 displayed total highly identifiable capillary loops growing into strands involving the entire optic disk and all

of the medullary rays. Finally, grade +5 described those eyes with NV complicated by hemorrhage and/or retinal detachment. Hemorrhaging occurred generally after grade +4 had been reached. In summary, stages of NV were graded as +1 (preproliferative), +2 (subtle NV), +3 (active NV), +4 (total NV), and +5 for NV with hemorrhage and/or retinal detachment.

#### Statistical methods

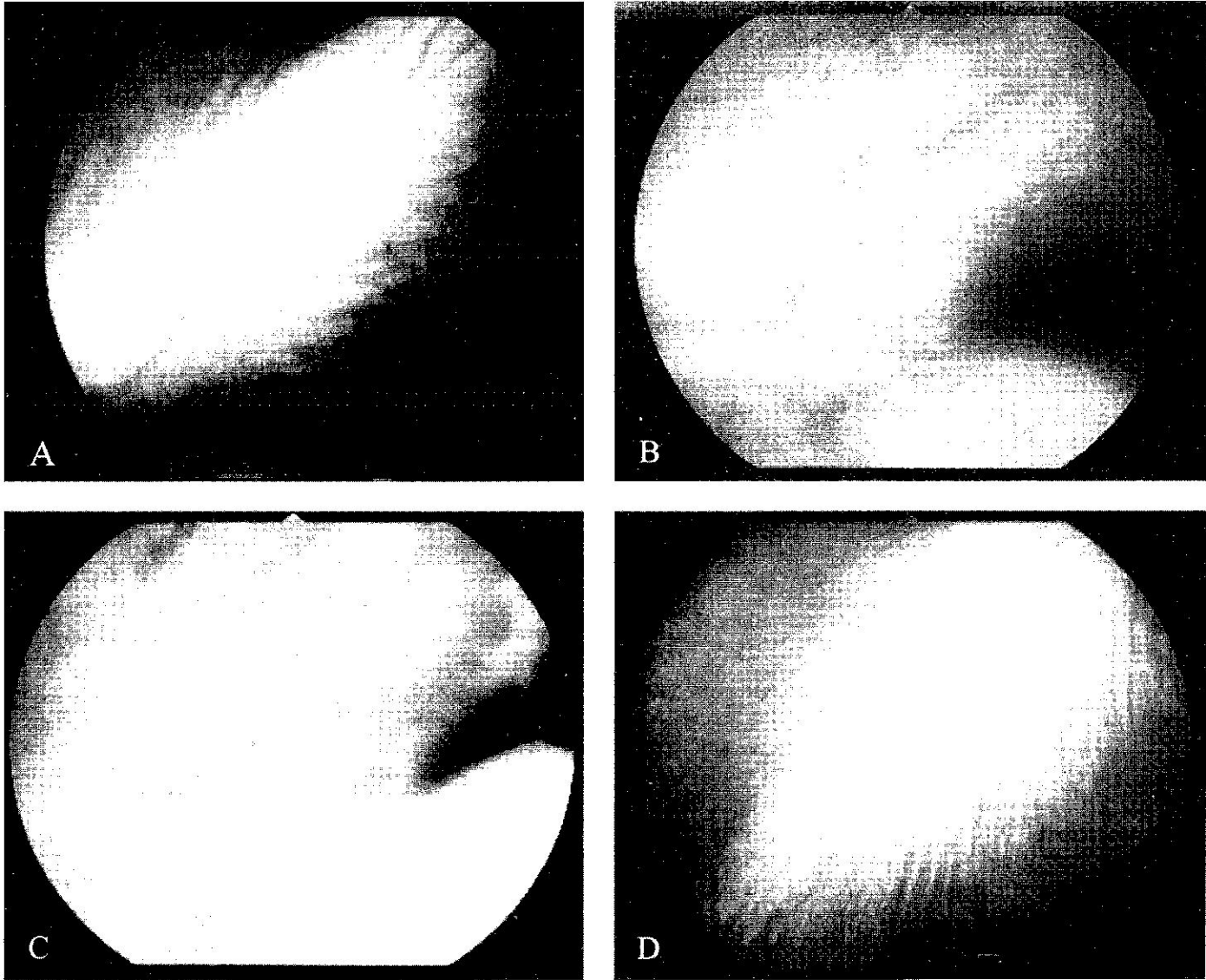
Data were analyzed using a repeated measures analysis of variance model with two grouping factors, breed of rabbit (NZW/BSC vs. Dutch belt) and pellet type (blank vs. VEGF/bFGF), and one within factor (days after implantation). The dependent variable was the NV grade of angiogenesis. The significance of main effects and interactions was measured by an F test.

#### Results

Although both breeds of rabbits are highly pigmented, the resulting retinal neovascular response was quite different after sustained intravitreal exposure to both VEGF and bFGF. In the Dutch belt rabbits (N = 7), NV grading of the angiogenic response demonstrated either +4 with hemorrhaging or +5 with complications of retinal detachment by day 20 after implantation. After hemorrhaging with or without retinal detachment, the lesions remained stable between day 20 and day 30. Figure 1 displays at both day 6 (Fig. 1B) and day 10 (Fig. 1C) a grade of +3 and +4, respectively, for the Dutch belt rabbits. In contrast, the NZW/BSC animals (N = 5) gave a more muted response with a maximum NV grade of +2 following exposure to the same amount of VEGF and bFGF (Fig. 2B). Control eyes that received only blank (PBS solution) pellets displayed no evidence of retinal NV in either the Dutch belts (N = 5) or the NZW/BSC rabbits (N = 5) at all time points examined over the 30-day period.

Results of statistical analysis for both the NZW/BSC and Dutch belt animals (Fig. 3) show a significant increase in angiogenesis with the VEGF/bFGF pellet compared to the blank pellet ( $p = 0.037$  and  $p < 0.00005$ , respectively). A significant interaction effect for breed and pellet type was also observed ( $F = 44.85$  with 1 df,  $p < 0.00005$ ), indicating a difference between the breeds in the angiogenic response to the pellet. The statistics confirm the qualitative clinical observations over the course of the 30-day study period.

Finally, the RPE pigmentation patterning above and below the optic streak appears different between the two breeds of pigmented rabbits. In turn, this pattern difference presented different views of the underlying choroidal vasculature. With the Dutch belt animals, the pigmentation was a consistent black with very little variegation. However in the NZW/BSC rabbits, the pigmentation had a highly mottled variegated pattern that allowed a more extensive view of the underlying choroidal vasculature.



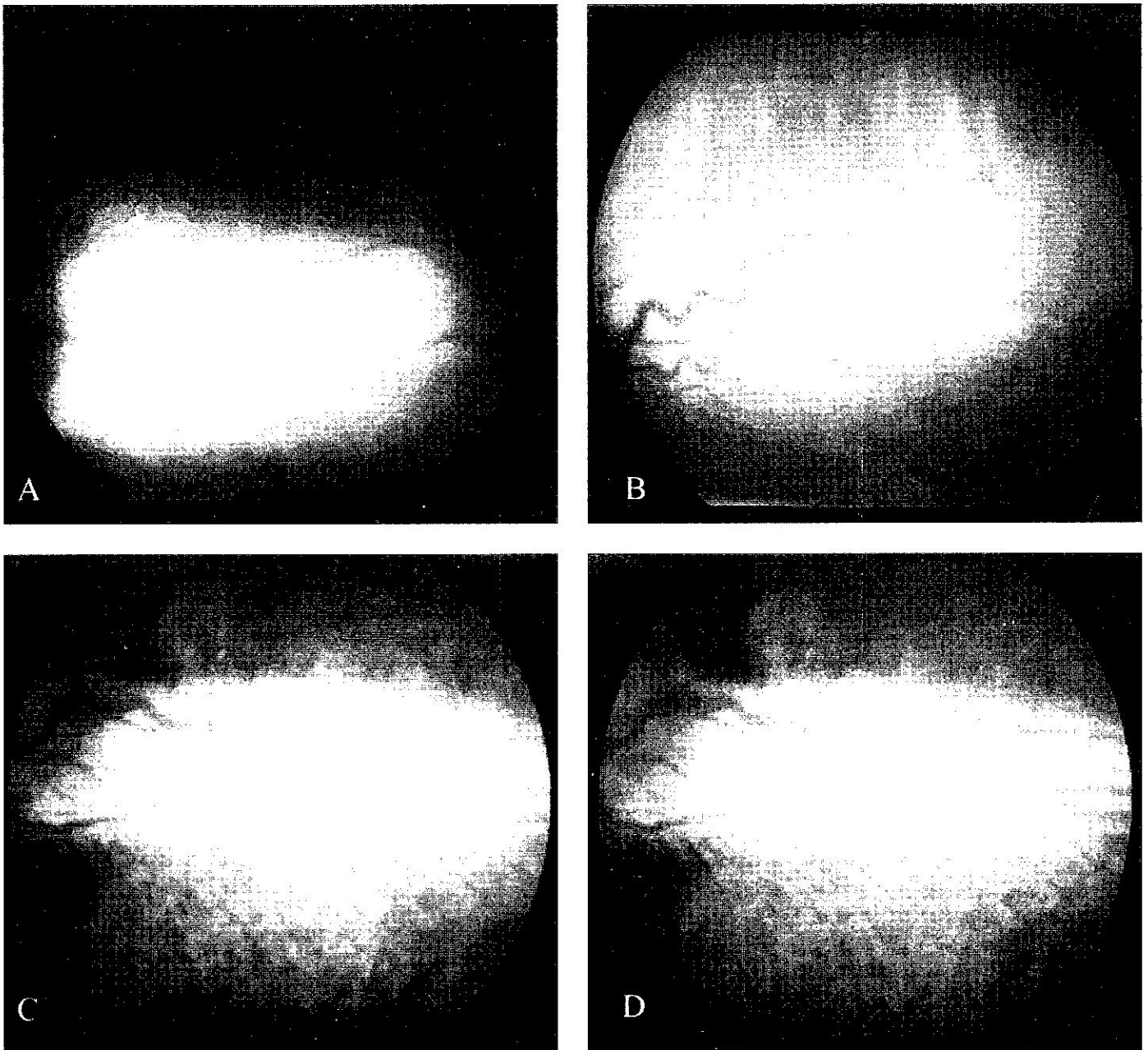
*Figure 1.* Stimulation of retinal neovascularization by simultaneous sustained intravitreal release of both vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) in Dutch belt rabbits. (A) Baseline fundus photograph prior to implantation of the Hydron pellet. (B) Same rabbit at 6 days after VEGF/bFGF pellet implantation. (C) Same rabbit at 10 days after VEGF/bFGF pellet implantation. (D) As a surgical control, a fundus photograph of another Dutch belt rabbit is shown at 26 days after implantation with a blank Hydron pellet.

## Discussion

This study demonstrates that the robust retinal vascular changes, subsequent hemorrhaging, fibrovascular membrane formation, and traction retinal detachment that occur in the pigmented Dutch belt rabbit after intravitreal implantation of sustained-release pellets containing both VEGF and bFGF is not present in the highly pigmented NZW/BSC rabbit. Neither sustained VEGF nor bFGF alone in the Dutch belt rabbits produces a retinal angiogenic response, although extremely high levels of sustained VEGF has been shown to yield a mild transient dilatation and tortuosity of existing blood vessels.<sup>9,10</sup> Since the Dutch belt animals did not yield an angiogenic response to either VEGF or bFGF,

implantation of sustained-release pellets containing either growth factor alone was not performed on the NZW/BSC rabbits.

Interestingly, the pigmentation patterning of the underlying retinal pigment epithelium (RPE) is subtly different between the two rabbit breeds that were utilized in this study. The NZW/BSC breed possesses a highly mottled variegated pigmentation pattern within the RPE while the Dutch belt animal displays a pigmentation pattern that is consistently dark with little mottling. Whether this subtle structural difference in pigmentation pattern of the RPE reflects a corresponding subtle functional difference is not known. Since RPE cells produce promoters that stimulate extracellular matrix contraction<sup>31</sup> and physically can pull collagen fibers

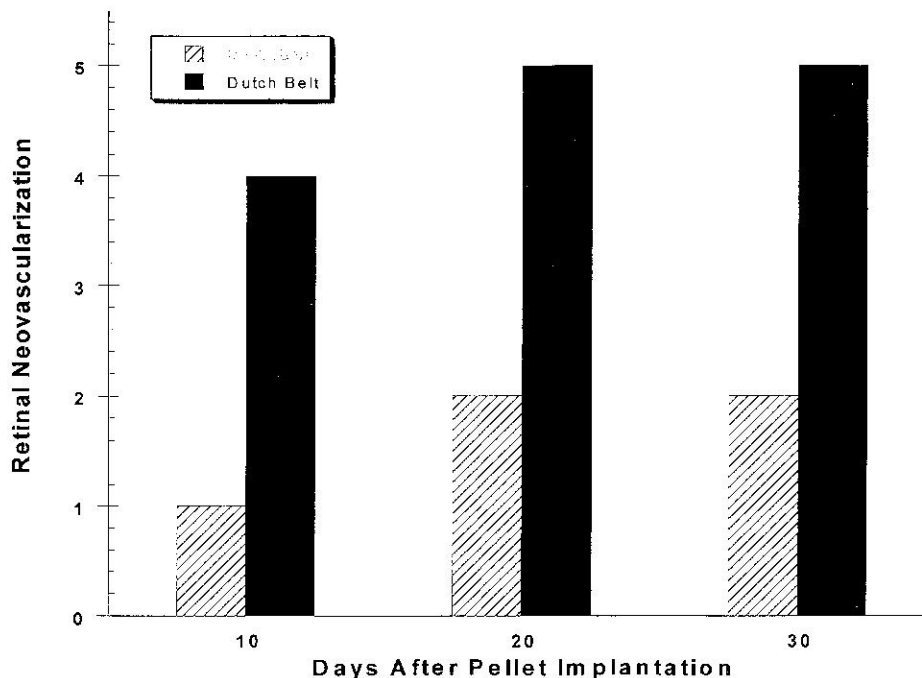


*Figure 2.* Time course of retinal vascular changes in the NZW/BSC animals after intravitreal implantation of sustained-release pellets containing both VEGF and bFGF. (A) Baseline fundus photograph prior to implantation of the non-biodegradable Hydron pellet. (B) Same rabbit at 20 days after VEGF/bFGF pellet implantation. (C) As a surgical control, fundus photograph is shown of a NZW/BSC rabbit prior to implantation; and (D) at day 20 after implantation.

toward themselves,<sup>32, 34</sup> these cells can act as both promoters and active components of traction retinal detachment<sup>35, 36</sup> within the vitreal cavity.

Differences in RPE pigmentation patterning may indicate subtle differences in RPE-related functions that could influence the rate and course of disease progression for diabetic retinopathy. Such subtle physical and functional differences may be a reflection of the genetic heterogeneity of angiogenesis at different biochemical and molecular levels where important factors and their respective receptors may differ.

Recent identification of a tissue-specific regulation of angiogenesis for promoting specifically endocrine-derived gland cells<sup>22</sup> suggests that additional tissue-specific angiogenic regulators may exist in other tissues for site-specific regulation of endothelial cell growth and differentiation. Uncovering a corresponding retinal and RPE-specific regulator of angiogenesis may provide clues for novel therapies of posterior segment neovascular diseases such as proliferative diabetic retinopathy and AMD. Interestingly, the tissue-specific pigment epithelium-derived factor has been shown to be a



**Figure 3.** Graphical representation of retinal NV on different days after intravitreal implantation of sustained-released pellets containing both VEGF and bFGF in either the Dutch belt or NZW/BSC rabbits. A significant difference ( $F = 44.85$  with 1 df,  $p < 0.00005$ ) is seen between the pigmented breeds in the retinal angiogenic response to sustained intravitreal VEGF and bFGF. For both the NZW/BSC and Dutch belt animals, a significant increase in angiogenesis occurred with the VEGF/bFGF pellet in comparison to control blank pellet ( $p = 0.037$  and  $p < 0.00005$ , respectively).

potent inhibitor of angiogenesis<sup>37</sup> and is able to inhibit both experimental retinal and choroidal NV.<sup>38,39</sup>

Overall, the results of this study indicate that the angiogenic response of the retina to exogenous growth factors is different between two breeds of pigmented rabbits. Another study that was reported in the literature utilized NZW rabbits and demonstrated that with high levels of VEGF the angiogenic response was fairly weak and regressed spontaneously.<sup>10</sup> Future *in vivo* studies with animals that involve either inducing angiogenesis with exogenous growth factors or testing anti-angiogenic agents should be performed with breeds that are consistent in response. As the population ages in the United States, the number of people with diabetes and complications of diabetes will increase dramatically. Understanding the early molecular processes that are involved in the pathogenesis of proliferative diabetic retinopathy<sup>40,41</sup> will provide novel pathways for the rational design of highly selective therapeutic agents in preventing severe visual loss.

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