Effect of Galactose Diet Removal on the Progression of Retinal Vessel Changes in Galactose-Fed Dogs

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PURPOSE. Feeding dogs a diet containing 30% galactose induces experimental galactosemia and results in the formation of diabetes-like microvascular lesions of the retina. The appearance and progression of these retinal lesions can be arrested in a dose-dependent manner by treating these dogs with aldose reductase inhibitors from the onset of galactosemia. To determine whether the elimination of galactosemia can also reduce the progression of retinal lesions, the galactose diet was removed from the galactosemic dogs after either the appearance of pericyte ghosts or formation of microaneurysms.

METHODS. Ten control dogs were fed a normal diet, and 50 dogs were fed a diet containing 30% galactose. The galactose diet was removed from 15 dogs after 24 months, the time at which pericyte ghosts had previously been observed to develop, and from another 15 dogs after 31 months, when microaneurysms had previously been observed to develop. Eighteen dogs were continued on a galactose diet. Beginning at 24 months, eyes from each group were enucleated at approximately 6-month intervals. Changes in retinal lesions were quantified by computer image analyses.

RESULTS. Significant (P < 0.05–0.01) increases in the endothelium-pericycle (EP) ratio and decreases in pericyte density were observed with increased duration of galactose feeding. Although no reversal of retinal lesions occurred, differences in the progression of retinal lesions between the galactose-fed and galactose-deprived groups became evident after 12 to 24 months.

CONCLUSIONS. Discontinuation of galactose in the diet at the initial stages of background retinopathy beneficially delays the progression of retinal lesions. (Invest Ophthalmol Vis Sci. 2002;43:1916–1921)

Diabetic retinopathy, a leading cause of blindness, is a major long-term complication of diabetes mellitus that is characterized by vascular changes of the retinal capillary bed. In its early, nonproliferative stage, these changes include the formation of microaneurysms, intraretinal hemorrhages, exudates and altered blood flow. In the later, proliferative stage, neovascularization occurs as retinal vessels break through the inner limiting membrane and grow into the vitreous.1 A hallmark of this disease is the destruction of pericytes (mural cells) from retinal capillaries and the formation of pericyte ghosts.2,3 Their loss is associated with vessel dilation, formation of microaneurysm, and endothelial cell proliferation.

Retinal vessel changes similar to human retinopathy have been observed to occur in diabetic4–6 and galactose-fed7–9 dogs. The degeneration of retinal capillary pericytes to form pericyte ghosts is the earliest morphologic change observed in these dogs. This is followed by the appearance of microaneurysms, localized capillary acellularity in which both pericytes and endothelial cells have degenerated, retinal capillary occlusions, and the eventual formation of large acellular capillary beds. Subsequently, in the galactose-fed dogs, advanced retinal changes associated with retinal ischemia, including intraretinal neovascularization and intravitreal retinal vascular growth, also appear.

Galactosemia is associated with a number of diabetes-like lesions.10 Galactosemia, both hereditary and experimentally induced with galactose feeding, is associated with the rapid formation of galactitol and, to a lesser extent, the formation of galactonate acid and/or galactonolactone.11–14 Long-term galactosemia is also associated with increased nonenzymatic glycation, basement membrane thickening, and biochemical changes that include decreased glutathione, tauirine, and myoinositol levels, decreased adenosine triphosphate (ATP) and amino acid transport activity, increased PKC and VEGF levels, and altered membrane permeability. Formation of galactitol is catalyzed by the enzyme aldose reductase, and its intracellular accumulation precedes the biochemical changes associated with long-term galactosemia. The administration of aldose reductase inhibitors to animals from the onset of galactose feeding has been observed to reduce galactitol formation and prevent the subsequent formation of diabetes-like lesions.10 However, aldose reductase inhibitors do not inhibit gulonic acid or formation of galactonolactone, suggesting that the formation of these latter two metabolic intermediates, unlike galactitol, do not contribute to the formation of diabetes-like lesions.14

Studies using the galactose-fed dog model indicate that degeneration of retinal capillary pericytes is linked to the aldose reductase-catalyzed production of galactitol and that their loss precedes further vascular changes associated with retinopathy. In beagles fed a 30% galactose diet for 36 to 38 months, the onset and progression of destruction of pericytes and the formation of microaneurysms was reduced by the concomitant administration of the aldose reductase inhibitors sorbinil (5-6-fluoro-spirochroman-4,5-imidazolidine-2,4-dione), its more potent 2-methyl analogue M79175 (2-methyl-6-fluoro-spirochroman-4,5-imidazolidine-2,4-dione), or a combination of both inhibitors.15–18 The dose-dependent reduction in retinal vessel changes by aldose reductase inhibition was confirmed by the quantification of retinal vascular changes, by computer-assisted image analysis.19,20 Kern and Engerman21 observed formation of galactitol in isolated canine retinal capillaries incubated in vitro with 30 mM galactose, and this formation was inhibited by the aldose reductase inhibitor sorbinil. Although Kern and Engerman22 failed to detect aldose reductase in isolated capillaries immunohistochemically, Akagi et al.23 demonstrated immunohistochemically the presence of...
aldose reductase in pericytes but not endothelial cells in canine retinal capillaries isolated by trypsin digestion. Canine retinal capillary pericytes cultured in vitro in high glucose- or galactose-containing medium form sorbitol or galactitol, respectively, and aldose reductase is expressed by these cells.\textsuperscript{24–26} Furthermore, formation of galactitol in the cultured pericytes has been observed to induce apoptosis, and both formation of galactitol and the subsequent induction of apoptosis were inhibited by the presence of aldose reductase inhibitors.\textsuperscript{24–26} Expression of aldose reductase was not detected in canine retinal capillary endothelial cell cultures, and the cells failed to convert glucose or galactose to sorbitol and galactitol, respectively. Moreover, culturing these cells in galactose-containing medium did not induce apoptosis.\textsuperscript{26}

Although experimental evidence clearly demonstrates that aldose reductase inhibitors administered from the onset of galactose feeding can inhibit degeneration of pericytes and subsequent retinal vascular changes associated with diabetic retinopathy in a dose-dependent manner, the effects of removal of galactose from the diet on the progression of retinal changes is less clear. In galactose-fed animals, removal of galactose results in a cessation of galactosemia and decreased polyol pathway activity.\textsuperscript{27} In the current study, the effect of discontinuation of galactose on degeneration of pericytes and retinal capillary changes in dogs after 24 and 31 months of galactose feeding were investigated and compared with retinal changes in similar dogs continued on a galactose diet for up to 60 months. The 24- and 31-month periods correspond to the early stages of retinopathy, when formation of pericyte ghosts and microaneurysms, respectively, have been observed to occur.\textsuperscript{8,15,16}

METHODS

Dogs

Experiments conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Dogs were 9-month-old male beagles (Marshall Farms USA Inc., North Rose, NY) individually housed in 0.9 × 2.7-m runs and fed a daily diet (Bioserve, Frenchtown, NJ) consisting of approximately 450 g of standard dog chow containing either 30% nonnutrient fiber or 30% galactose, as previously described.\textsuperscript{13} Ten dogs received the control diet containing 30% fiber, and 50 dogs received the galactose diet. After 24 months, the galactose was removed from the diets of 15 randomly selected dogs in the galactose-fed group and at 31 months from the diets of an additional 15 randomly selected dogs in the galactose-fed group.

Enucleations in any given period were limited to one eye of each dog, so that all eyes in each group at the specified time points were from separate animals. In each group, dogs with two eyes underwent enucleation first, followed by dogs with one eye. All dogs were killed after the second enucleation. Based on this criterion, dogs were randomly selected for enucleation as follows: control group: two eyes at 24 months and three eyes at 30, 36, 42, 48, 55, and 60 months; galactose-fed group: two eyes at 24 months, five eyes at 30 and 36 months, six eyes at 42, 48, and 55 months, and three eyes at 60 months; 24-month reversal group: four eyes at 24 months, five eyes at 30 and 36 months, and four eyes at 42, 48, 55, and 60 months; and the 31-month reversal group: five eyes at 24, 30, 36, 42, 48, 55, and 60 months. Baseline data for the 31-month reversal group were obtained at the 30-month enucleation period, 1 month before removal of galactose from the diet.

Clinical blood chemistry profiles, conducted at 3-month intervals, included determinations of complete blood counts with differential, serum T3, thyroxine (T4), glucose, serum urea nitrogen, creatinine, sodium, potassium, chloride, calcium, phosphorus, uric acid, total proteins, albumin, globulin, aspartate aminotransferase, alanine aminotransferase, 2-glutamyl transferase, alkaline phosphatase, lactate dehy-

drogenase, total bilirubin, direct bilirubin, triglycerides, cholesterol, and glycosylated hemoglobin. Hb A1c levels were obtained by the high-pressure liquid chromatography (HPLC) method at 3-month intervals. All tests were conducted by Maryland MedPath (Rockville, MD).

No significant abnormalities in blood chemistry profiles were observed.

Preparation of Retinal Vessels

Enucleated eyes were fixed in 4% paraformaldehyde that was dissolved in 0.1 M phosphate buffer (pH 7.4) and the anterior segment (lens, cornea, and iris) was removed. After a 3-hour fixation, the retinas were isolated from the sclera and carefully separated and removed from the choroid. After a 5- to 7-day fixation in the paraformaldehyde solution, the intact isolated retina was washed with phosphate buffer and digested with gentle agitation at 37°C for 10 to 20 minutes with 3% trypsin (1:250; Difco Laboratories, Detroit, MI) in 0.1 M Tris buffer (pH 7.8). The isolated retinal vasculature was then placed on 1% gelatin-coated glass slides, air dried, washed with water, and stained for 10 minutes with periodic acid-Schiff-hematoxylin (Sigma Chemical Co., St. Louis, MO). The stained retinal vessels were dehydrated with graded ethanol solutions (50%, 75%, 85%, 95%, and 100%), immersed three times in xylene, and mounted with xylene-soluble permanent mounting medium (Permount; Fisher Scientific, Fairlawn, NJ).

Quantitative Analysis of the Retinal Vasculature

Quantitative analyses were conducted as previously described.\textsuperscript{14,15} Briefly, enlarged photographs of each isolated retinal vessel preparation were placed on a digitizing tablet (GTCO; Rockville, MD) and properly aligned according to the location of the retinal vessels. Starting at the 12 o’clock (0°) position, the x, y coordinates of the ora serrata and circumference of the optic disc were obtained by tracing the outer edge of the retinal vessel preparation and optic disc over the digitizing tablet. Next the location of each retinal cut made to flatten the retina onto the glass slide was defined by entering the coordinates on each outside edge of the cut and the innermost extent of the cut. From these coordinates, retinal maps containing 24 sectors were constructed by computer. In these maps, the area encompassed between 1 disc diameter outside the optic disc and 1 disc diameter inside the ora serrata was divided into three annuli of equal widths. Then eight meridians at 0°, 45°, 90°, 135°, 180°, 225°, 270°, and 315° from the center of the optic nerve were generated to form 24 sectors, and the geometric midpoints of each sector was then determined. A transparent overlay of each retinal map, reduced to the original size of each retinal spread, was then used as the template for regional analysis. As illustrated in Figure 1, regional analysis of the retinal vasculature
was conducted at the geometric midpoints of 12 subregions corresponding to areas where a greater than 10% incidence of microaneurysms had been observed. At each of the 12 midpoints, four adjacent color images were acquired with a charge-coupled device (CCD) color camera attached to a microscope (model M-859; Leitz, Wetzlar, Germany). Analysis in the 48 color images from each retina (approximately 1.2 mm²) was restricted to capillaries. Vascular cells (endothelial cells and pericytes) were classified after microscopic observation and marked on each image. Capillary length and area were manually recorded using the digitizing program of an image analysis system (Cue-3; Olympus, Tokyo, Japan). Capillary areas were computed by tracing both sides of the capillary walls. Acellular capillaries were also included in these measurements. Results in each retina are expressed as the mean of data collected from all 12 subregions. Calculations and statistical analyses were conducted on the NIH Prophet computer system using the Newman-Keuls multiple range and Kruskal-Wallis test for significance.

RESULTS

Nine-month-old male beagles were fed a diet containing either 30% nonnutrient filler (control group) or 30% galactose. After 24 months of galactose feeding, the period required for formation of pericyte ghosts, the galactose was removed from the diets of 15 randomly selected dogs (24-month reversal group). Galactose was removed from the diets of an additional 15 randomly selected dogs after 31 months, the period when microaneurysms form (31-month reversal group). Eyes were periodically enucleated in dogs from each group, and the intact retinal vasculature from each eye was isolated by trypsin digestion and subjected to regional quantitative analysis. The levels of galactosemia were monitored in each group by daily feeding records and quarterly determinations of Hb A₁C levels. As illustrated in Figure 2, the levels of Hb A₁C increased in dogs fed a galactose diet and decreased in the control dogs after the galactose was removed from the diet.

Mean measurements of pericytes per millimeter capillary length and endothelial cells per millimeter capillary length from the intact capillaries conducted at the geometric midpoints of 12 subregions, corresponding to areas where a greater than 10% incidence of microaneurysms had previously been observed are summarized in Figure 3. In the galactose-fed group a steady decline in pericytes per capillary length was observed in correlation with the duration of galactose feeding, whereas the levels of endothelial cells per capillary length did not significantly change until 60 months. Although pericyte density (cells per millimeter capillary length) appeared lower in the galactose-fed dogs compared with normal dogs after 24 months of galactose feeding, this difference was not observed to be significant until 42 months. In dogs in which the galac-
The diet was discontinued at either 24 or 31 months, the density of pericytes and endothelial cells remained constant from the time of reversal up to the 60-month period of examination. These results indicate that removal of galactose inhibited the further destruction of the pericytes. This marked reduction in loss of pericytes is more clearly seen when endothelial cell-to-pericyte (E-P) ratios in cellular capillaries are compared. As summarized in Figure 4, a steady increase in the E-P ratio occurred in dogs fed galactose for 24 to 55 months, with a dramatic increase at 60 months, whereas the E-P ratio in control, non-galactose-fed dogs remained constant (Fig. 3). In contrast, the E-P ratios for dogs with the galactose diet removed at either 24 or 31 months remained essentially constant from the time of reversal up to the 60-month period of examination.

In addition to destruction of pericytes, continued galactose feeding also resulted in an increase in the levels of acellular capillaries where both pericytes and endothelial cells degenerate. As illustrated in Figure 5, a steady increase in the percentage of acellular capillary vessels was observed after 40 months of galactose feeding, but this increase was not observed in the dogs with galactose removed from the diet.

Overall formation of microaneurysm in the retina was also monitored. As illustrated in Figure 6, the number of microaneurysms dramatically increased in the galactose-fed dogs. In contrast, formation of microaneurysms in the reversed groups was markedly reduced.

**DISCUSSION**

Experimental galactosemia, which activates the polyol pathway, has been used extensively to explore the pathogenesis of diabetic complications. The galactose-fed dog is an animal model that both histologically and clinically demonstrates retinal vascular changes associated with diabetic retinopathy. These changes progress from an initial aldose reductase-associated destruction of the pericytes to form pericyte ghosts to the formation of microaneurysms, preretal and intravitreal hemorrhages, acellular vessels, occluded vessels, and large areas of nonperfusion, intraretinal microvascular abnormalities, peripapillary neovascularization, and intravitreal retinal vascular growth. Destruction of pericytes has been linked to the abnormal accumulation of excess sugar alcohols, sorbitol, and galactitol under hyperglycemic and galactosemic conditions, respectively. Several prevention studies have demonstrated that adequate administration of aldose reductase inhibitors can reduce degeneration of pericytes and the subsequent formation of microaneurysms and hemorrhages in a dose-dependent manner in galactose-fed dogs.

Many investigators use galactose-fed animals as models for investigating the specific role of aldose reductase in the formation of microaneurysms, preretal and intravitreal hemorrhages, acellular vessels, occluded vessels, and large areas of nonperfusion, intraretinal microvascular abnormalities, peripapillary neovascularization, and intravitreal retinal vascular growth.
tion of these diabetes-like lesions, because galactose is rapidly metabolized to galactitol by aldose reductase. 10–29 Experimental studies indicate that the intracellular accumulation of galactitol is a primary biochemical event observed in tissues possessing aldose reductase and that this is followed by a cascade of biochemical changes that eventually result in the formation of diabetes-like lesions. In tissues possessing aldose reductase, removal of galactose from the diet can result in a rapid decrease in galactitol formation and a potential reversal of subsequent biochemical changes. 27 The present study indicates that the progression of vascular retinal changes associated with diabetic retinopathy can also be ameliorated by removal of galactose from the diet during the early stages of retinopathy, when formation of pericyte ghosts and microaneurysms occurs.

Determining efficacy in the amelioration of retinal vascular changes by agents administered from the onset of galactose feeding is relatively straightforward, because in protection studies the onset of the complication and its progression provide clear end points. Assessing similar amelioration in intervention studies is more complex, however, because the actual progression of retinal vascular changes must be assessed. This was accomplished in the present study through the use of computer-assisted methods for quantifying the observed retinal changes that allowed us to measure retinal changes in similar locations in the isolated intact retinal vasculature from each different dog. The development of this method and its validation with aldose reductase inhibitor–treated dogs are described and discussed elsewhere. 19,20 Biological variations in the progression of retinal changes were minimized by using beagles of the same sex, age, and supplier as in previous experiments.

Studies of diabetic and galactosemic animals indicate that many of the lesions associated with diabetes or galactosemia become irreversible after a certain time. 50 For example, sugar-induced cataracts are only reversible when they are in the early vesicular stage, 25 and retinopathy in the rat cannot be arrested once significant basement membrane thickening and destruction of pericytes have occurred. 51 In retinal vessels, lesions such as loss of pericytes and the formation of microaneurysms and acellular vessels are all irreversible. Potential intervention studies in retinopathy are confounded, because the point of no return in the progression of retinal changes is unknown. Because it is known that intervention in the early stages of diabetic complications is most beneficial, 52,53 the galactose diet was removed at the early stages of retinopathy when the histologic appearance of pericyte ghosts (24 months) and microaneurysms (31 months) occurs. Because it could be anticipated that intervention would result in only a gradual decrease in the progression of retinal changes, retinal changes in the eyes of these dogs were serially examined through enucleations performed at approximately 6-month intervals, and the progression was compared with that in similar dogs maintained on either the galactose or control diet throughout the duration of the study. This allowed us to identify clearly the progression in each group.

Based on the experimental observations that galactosemia is linked to formation of galactitol in the retinal pericytes and that formation of galactitol is associated with destruction of pericytes, the present study clearly indicates that removal of galactose from the diet during the early stages of formation of pericyte ghosts or microaneurysms is beneficial in decreasing the progression of retinal degeneration of pericyte, as indicated by measurements of E-P ratios (Fig. 5) and the number of microaneurysms present (Fig. 6). A similar beneficial effect in the formation of acellular capillaries was also observed (Fig. 5). These results also confirm that galactose feeding results in the selective destruction of pericytes (Fig. 4) and that destruction of pericyte precedes the formation of microaneurysms and areas containing acellular capillaries. Acellular capillaries correspond to the areas of nonperfusion, 18 which are associated with hypoxia.

Tight control of hyperglycemia has been shown to be beneficial in reducing the progression of diabetic retinopathy, both in humans 32 and in diabetic dogs. 55 Aldose reductase–catalyzed sorbitol levels are directly proportional to the levels of hyperglycemia, and tight control of hyperglycemia is associated with reduced polyol pathway activity. 54 Similarly, galactitol levels are directly proportional to the levels of galactosemia associated with the amount of ingested galactose. 10 In the present study the progression of diabetic retinopathy was also reduced when galactosemia and presumably polyol pathway activity were reduced by the removal of galactose. However, the present study contradicts the conclusion by Engerman and Kern 57 that removal of galactose from the diet does not halt the progression of retinopathy. In their limited study, four dogs of mixed age (1.5–2.5 years old) were fed a 30% galactose diet for 24 months. After enucleation of one eye in each dog, the dogs were fed a normal diet for 36 months, and the remaining eye was then enucleated. A wedge of nasal retina was removed from each enucleated eye, and the vasculature from each remaining retina was isolated by trypsin digestion. Based on subjective comparison of the midretina from the partial retinal vasculature of each of the four eyes versus the contralateral eye after 36 months of normal diet, Engerman and Kern reported increases in the amounts of acellular capillaries per 10 mm2, pericyte ghosts per 1000 cells, microaneurysms per eye, capillary basal lamina, and sudanophilic staining 36 months after removal of galactose from the diet. However, all these parameters used to define retinopathy in their dogs were lower in those with the galactose removed from the diet than in a separate study in which dogs were continued on the galactose diet for an additional 36 months. The major limitations of their study were the small number of animals; 5 the high variability of their results, with coefficients of variation of 46%, 93%, and 112% for pericyte ghosts, acellular capillaries, and microaneurysms, respectively; and that no animals were maintained on the galactose diet throughout the study for direct comparison. Moreover, Engerman and Kern 57 based their interpretations on the premise that capillary acellularity is the earliest histologic abnormality in dogs and that it can occur without loss of pericytes.

In our serial studies in beagles, with and without treatment with an aldose reductase inhibitor, we have documented that destruction of pericytes is the earliest histologic change and that it precedes capillary acellularity. In counting pericyte ghosts, we have previously demonstrated that E-P ratios or counts of pericytes per capillary length are less prone to error than counts of pericyte ghosts per 1000 cells. 20 Basement membrane thickening was not assessed in this study, because previous preliminary studies indicated that formation of pericyte ghosts precedes significant thickening of basement capillary membrane in the galactose-fed dog. This is in contrast to studies in rats in which significant basement membrane thickening occurred before or simultaneously with destruction of pericytes. 29

In the present study, little difference in the progression of retinal changes was observed between the removal of galactose in the diet at 24 and 31 months. This suggests that the point of no return associated with irreversible retinopathy was not reached in these studies. If one accepts the premise that removal of galactose from the diet is associated with decreased polyol pathway activity in tissues containing aldose reductase, then these findings complement those in previous prevention studies 15 in which inhibition of aldose reductase in galactose-fed dogs resulted in dose-dependent protection of retinal cap-
illary pericytes from destruction and from the subsequent formation of microaneurysms.

References