Altered Proliferation of Retinal Microvascular Cells on Glycated Matrix

Theodosia A. Kalfa,* Mary E. Gerritsen,† Edward C. Carlson,‡ Aaron J. Binstock,* and Effie C. Tsilibary*

Purpose. To investigate the effect of nonenzymatic glycosylation (glycation) of basement membranes (BM) and isolated BM proteins on the growth of retinal pericytes and retinal endothelial cells.

Methods. Type IV collagen, laminin, Engelbreth–Holm–Swarm tumor basement membrane (EHS–BM) and bovine retinal basement membrane (RBM), after incubation in the presence of reducing sugars to induce glucose-mediated modifications, or in the absence of any sugar (control), were used as a substrate to culture bovine retinal microvascular cells. Cell growth on the nonenzymatically glycylated and the corresponding control substrates was measured daily, using an automated cell counter.

Results. Retinal pericytes seeded on glycated type IV collagen proliferated consistently more slowly than on control type IV collagen \((P = 0.02)\), showing a 20% to 33% decrease throughout most of the growth curve, whereas on glycated laminin the difference from control was not significant. In contrast, proliferation increased by 16% to 25% for retinal endothelial cells on glycated laminin compared with control substrate \((P = 0.025)\), whereas on glycated type IV collagen the growth curve was not significantly different from the curve for the control. When seeded on whole glycated EHS-BM or RBM, proliferation of pericytes decreased by 20% to 30% \((P = 0.04)\); the endothelial cells showed no difference on glycated EHS-BM, however, the growth rate increased on glycated RBM by 25% to 30% more than it did for the control \((P = 0.01)\).

Conclusions. Nonenzymatic glycosylation of intact BM or individual BM macromolecules resulted in reduced proliferation of retinal pericytes and increased proliferation of retinal endothelial cells. These in vitro observations resemble some of the pathologic changes of the retinal microvascular cells observed in situ, when diabetic retinopathy develops. Invest Ophthalmol Vis Sci. 1995;36:2358–2367.

Diabetic retinopathy is among the most common diabetic complications and one of the leading causes of vision loss in industrialized countries. The prevalence of diabetic retinopathy is strongly associated with the duration of diabetes. Epidemiologic studies have concluded that ophthalmologically apparent retinopathy develops in 50% to 98% of persons with diabetes 15 years after the diagnosis of diabetes.1,2

The characteristic histopathologic processes of diabetic retinopathy are basement membrane (BM) thickening, pericyte degeneration, formation of microaneurysms, and focal capillary closure and acellularity, followed by abnormal proliferation of the endothelial cells. Endothelial proliferation leads to neovascularization of proliferative retinopathy.3–5

The recent results of the Diabetes Control and Complications Trial6 suggest that chronic hyperglycemia, accompanied by increased glycosylated hemoglobin values, directly correlates with the risk for developing retinopathy. The mechanism(s) that link hyperglycemia to the pathologic features of diabetic retinopathy have not been elucidated, but two such mechanisms are being investigated: intracellular hyperglycemia with disturbances in the polyol pathway7 and nonenzymatic glycosylation of long-lived proteins.8,9

From the *Department of Laboratory Medicine and Pathology, University of Minnesota Medical School, Minneapolis; the †Bayer Corporation, West Haven, Connecticut; and the Department of Anatomy and Cell Biology, School of Medicine, University of North Dakota, Grand Forks.

Supported by grants JDF 190361, JDF 132043, ADA; and NIH grant DK43574 to Dr. Tsilibary.

Submitted for publication August 30, 1994; revised April 19, 1995; accepted August 7, 1995.

Proprietary interest category: N.

Reprint requests: Effie C. Tsilibary, Department of Laboratory Medicine and Pathology, University of Minnesota Medical School, Box 609 UMHC, 420 Delaware Street SE, Minneapolis, MN 55455.

Copyright © Association for Research in Vision and Ophthalmology
All reducing sugars, including D-glucose and D-galactose, can initiate nonenzymatic glycosylation of proteins. This reaction involves the nonenzymatic condensation of a sugar aldehyde or ketone with a free amino group (often the ε-amino group of lysine residues) to form reversibly a Schiff base. The Schiff base rearranges to the more stable Amadori product, which is thermodynamically stable and are expected to accumulate onto long-lived biologic macromolecules, such as hemoglobin and the proteins of the extracellular matrix (e.g., collagen). The rate of this reaction increases with the glucose concentration.

The incidence and severity of diabetic retinopathy has been associated with high levels of nonenzymatically glycosylated hemoglobin, and has also been directly correlated to the values of collagen-linked fluorescence (fluorescence monitors the formation of AGE products). Moreover, it was shown recently that treatment of diabetic rats with aminoguanidine, an inhibitor of AGE product formation, prevents abnormal endothelial cell proliferation and pericyte dropout in the retina and inhibits the development of diabetic retinopathy.

In this report, we induced nonenzymatic glycosylation and AGE formation to the whole complex of bovine retinal basement membrane (RBMs) and Engelbreth-Holm-Swarm tumor basement membrane (EHS-BM), or to isolated molecules of type IV collagen and laminin, by in vitro incubation in the presence of reducing sugars. Then we used these BMs or BM components as an immobilized substrate to grow bovine retinal pericytes (BRP) or retinal endothelial cells (BRE), and we examined the effect of substrate glycation on the growth of these two cell types in culture.

**MATERIALS AND METHODS**

We adhered to the guidelines established in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Isolation Procedures**

Type IV collagen or laminin, major components of BMs, were purified from EHS tumor grown subcutaneously in mice that were made lathyritic by adding 0.1% β-aminopropionitrile to their drinking water, according to protocols previously described.

Fragments of whole EHS-BM were prepared with the following procedure. Aliquots of tumor, previously stored at −80°C in hypertonic buffer (50 mM Tris-HCl, 3.4 M NaCl, pH 7.4), containing 1 mM ethylenediaminetetraacetic acid (EDTA) and phenyl-methyl-sulfonylfloride (PMSF), p-hydroxymercuribenzoic acid, and N-ethylmaleimide (NEM) in a final concentration of 50 μg/ml each, as protease inhibitors) were thawed and solubilized in four volumes of the same buffer. The mixture was homogenized in a blender with two 30-second bursts of a high-speed blender. The homogenate was washed six times with the cold hypertonic buffer by repeated centrifugation (3750 rpm x 30 minutes at 4°C) and resuspension to remove serum and cellular proteins, and then it was washed four more times in cold isotonic buffer (50 mM Tris-HCl, 150 mM NaCl, 50 μg/ml PMSF, p-hydroxymercuribenzoic acid, and NEM, pH 7.4). The final pellet was resuspended in 10 volumes of cold phosphate buffer (0.2 M NaH2PO4·Na2HPO4, 0.02% NaN₃, 50 μg/ml PMSF, 50 μg/ml NEM, pH 7.4) and the solution, divided in 10 ml aliquots, was sonicated 12 times for periods of 10 seconds, on ice, with the microtip of a model 250/450 sonifier (Branson Ultrasonics Corporation, Danbury, CT).

Retinal basement membrane was prepared from bovine retinas as previously described. Briefly, neuroretinas were removed from freshly enucleated bovine eyes, minced, and homogenized in a tissue-grinding vessel. The homogenate was poured successively over a 210-μm and an 88-μm mesh nylon sieve while washing with chilled saline to retain finally a vessel fraction highly enriched in capillaries. RBMs were prepared from this small vessel fraction by detergent extraction, after cell disruption by osmotic lysis: Vessels from 30 bovine eyes were suspended in a 50-ml tube of distilled water for several hours with gentle agitation. After a brief centrifugation, the pellet containing the vessels was collected and resuspended in 5% Triton-X-100 for 2 to 4 hours. Centrifugation was repeated, followed by resuspension and incubation for 1 to 2 hours in 40 ml of 1 M NaCl containing 2000 Kunitz units of DNase. This was recentrifuged and resuspended in a solution of 4% sodium deoxycholate for 4 hours. The final suspension was washed several times by repeated centrifugation and resuspension in distilled water to remove cellular membranes and debris. All of these solutions contained 0.05% NaN₃. All treatments were done at 4°C, except the sodium deoxycholate step, which was done at 22°C to prevent gelation.

The final pellet from the procedure just described, containing RBM, was suspended in 10 volumes of phosphate buffer (0.2 M NaH2PO4·Na2HPO4, 0.02% NaN₃, 50 μg/ml PMSF, 50 μg/ml NEM, pH 7.4) and divided in 10-ml aliquots stored frozen at −80°C. Each aliquot was sonicated 20 times for periods of 10 seconds on ice immediately before being used for an experiment.

**Nonenzymatic Glycosylation of BM Macromolecules and Whole BM**

The proteins were glycosylated in vitro with a slight modification of the protocol previously described by...
Haitoglou et al.: Laminin was dialyzed extensively against PBS, pH 7.4 containing 10 mM EDTA, 0.02% NaN₃, 50 μg/ml PMSF, and 50 μg/ml NEM at 4°C; type IV collagen was dialyzed against the same buffer with 0.5 M NaCl. The buffer used was designed to limit proteolytic degradation and polymerization. The protein solutions were centrifuged at 20,000 rpm for 20 minutes at 4°C in a Beckman L8-M ultracentrifuge to clear large aggregates, and they were incubated in the absence of sugar or in the presence of either 50 mM or 500 mM D-glucose for 72 hours at 29°C in the dark with occasional shaking. Under these conditions, approximately 1 mole and 10 moles of glucose were incorporated per mole of macromolecule, respectively. After extensive dialysis against PBS, pH 7.4, at 4°C and centrifugation at 10,000 rpm for 10 minutes at 4°C, samples were used to coat 96-well Immulon 1 plates (Dynatech Labs, Chantilly, VA).

EHS-BM and RBM fragments were nonenzymatically glycosylated by the following protocol: The sonicated BM in a concentration of 50 to 100 μg/ml in phosphate buffer (0.2 M NaH₂PO₄-Na₂HPO₄, 0.02% NaN₃, 50 μg/ml PMSF, 50 μg/ml NEM, pH 7.4) was incubated at 29°C for 6 to 15 days with either no sugar or 1 M D-ribose. Previous experiments used ribitol (adenitol) as an additional control and yielded similar results as the controls that were incubated in the absence of any sugar. Therefore, because of the limited availability of retinal BM, we did not use ribitol controls. Before the incubation, the solutions were degassed (before adding the protease inhibitors) under vacuum for 2 hours to remove dissolved O₂ and saturated with inert gas (argon) to avoid oxidation. After extensive dialysis against PBS, pH 7.4, at 4°C, samples were examined in a Perkin-Elmer (Norwalk, CT) LS50B luminescence spectrometer (fluorometer) at an excitation wavelength of 370 nm and an emission wavelength of 440 nm and used to coat 96-well Immulon 1 plates.

An additional experiment was performed by inducing glycation to previously coated BM. We first coated EHS-BM on plastic wells (of the 96-well Immulon 1 plates) by adding to each well 100 μl of a suspension containing 8 μg/ml EHS-BM in PBS and incubating overnight at 4°C. The next day we gently aspirated the solution, leaving the particles of EHS-BM bound onto the plastic, and we added 100 μl/well of degassed phosphate buffer containing either no sugar or 1 M D-ribose. We prepared six wells each for the control and glycated matrix in each of six plates. Then we placed the plates in sealed bags after substituting the air in the bag with argon, and we incubated them at 29°C for 10 days.

The concentration of the protein solution and sonicated BM suspension was determined measuring the absorbance at 215 and 225 nm by the Waddell method.

Iodination of EHS-BM and Testing of Coating Efficiency

To determine the percentage of BM fragments that remained bound to plastic, after coating the wells with BM suspension, we used the EHS-BM as a model for the various BM, because it is easier to attain large amounts of this BM-like matrix. EHS-BM was labeled with Na¹²⁵I (DuPont-New England Nuclear, Boston, MA) by chemical oxidation using chloramine T (Sigma Chemical Co., St. Louis, MO). More specifically, 2 ml of sonicated EHS-BM, prepared as described in the isolation procedures, of approximate concentration 100 μg/ml, in reaction buffer (50 mM Tris, 1.2 M urea, 0.15 M NaCl, 50 μg/ml PMSF, p-hydroxymercuribenzoic acid, and NEM, pH 7.4) was mixed with 4 mCi ¹²⁵Iodine and 50 μl chloramine-T solution (0.2 mg/ml in reaction buffer) and incubated for 5 minutes. The reaction was stopped by adding 100 μl of 0.1 M solution of NaHSO₃. The solution with the iodinated EHS-BM was dialyzed extensively against cold phosphate buffer to remove unbound Na¹²⁵I. Incorporated¹²⁵I in EHS-BM was estimated by trichloroacetic acid precipitation and found to be 80% of the total counts of a sample.

The labeled EHS-BM was mixed with unlabeled EHS-BM and divided in three equal parts that were incubated for 5 days at 29°C, in the absence of sugar or with 1 M ribitol or with 1 M ribose, with the same setting that was described for nonenzymatic glycosylation of BM fragments. After extensive dialysis against PBS, pH 7.4, at 4°C, samples were used in serial dilution to coat 96-well Immulon 1 plates, in triplicate. One hundred microliters of each dilution was added per well and allowed to incubate overnight at 4°C. The next day the solution was aspirated, the wells were washed twice with 100 μl/well PBS, pH 7.4, and the BM fragments that remained bound onto plastic were solubilized with 100 μl lysis buffer (0.5 M NaOH + 1% sodium dodecyl sulfate) and quantified in a gamma counter. The coating efficiency was calculated as the percentage of counts added to each well that remained bound to the well at the end of the experiment.

Coating Plates

Type IV collagen was used in the concentrations of 25 μg/ml and 100 μg/ml, whereas laminin was used in the concentrations of 100 μg/ml and 200 μg/ml, for the retinal pericytes and retinal endothelial cells, respectively. These concentrations were selected according to initial experiments designed to determine conditions for optimal adhesion of each different cell type to type IV collagen or laminin, respectively. Fifty microliters of each protein solution was added per well and allowed to dry overnight at 29°C. Under these conditions, 0.6 and 2.3 μg of type IV collagen or 2.3
and 4.5 μg of laminin (for the retinal pericytes and retinal endothelial cells, respectively) remained bound to each plastic well, as had been previously determined using radiolabeled proteins. The plates were used immediately or stored at 4°C for no longer than 2 weeks.

EHS-BM was coated in a concentration of 8 μg/ml, and RBM was coated in a concentration of 10 μg/ml. One hundred microliters of the BM suspension was added per well and incubated overnight at 4°C. The next day, 80 μl was aspirated from each well and the plates were used immediately for the experiment. As previously described, by using radiolabeled matrix, we estimated that 0.6 μg EHS-BM and 0.7 μg RBM remained bound to each plastic well under these conditions.

Sets of four or six wells were used for each control as well as glycosylated protein or BM, depending on the availability of each matrix and of each of the cell lines. Bovine serum albumin was coated in four wells in each plate, in similar concentration with the examined protein or BM, to operate as a control.

**Cells**

We used BRP and BRE cells that were isolated from bovine retinas, characterized, and selectively cultured, as previously described. Cells were cultured in 75-cm² and 25-cm² flasks (Falcon, Oxnard, CA), respectively. The BRE cells were initially cultured in flasks coated with 5 ml/flask of 100 μg/ml fibronectin to facilitate endothelial growth. Both cell lines were subsequently cultured eventually to plastic and were passed before reaching confluence. The media used were RPMI 1640 containing 10% calf serum (for the BRP and Dulbecco’s modified Eagle’s medium containing 20% fetal calf serum for the BRE cells), supplemented with 50 IU/ml penicillin and 50 μg/ml streptomycin. Sera, media, and antibiotics were purchased from Sigma Chemical Company.

The cells were released from the tissue culture flasks, for passaging and use in experiments, by washing twice with Versene (sterile PBS + 0.5 mM EDTA) (Gibco BRL, Grand Island, NY) followed by incubation with trypsin-EDTA (Gibco BRL) for 1 to 2 minutes at 37°C. The concentration of the trypsin solution used was 0.05% trypsin containing 0.53 mM EDTA for the BRP and 0.015% trypsin containing 0.16 mM EDTA for the BRE cells.

Cells between passages 5 and 13 were used for the experiments.

**Cell Proliferation Assays**

The protein or BM-coated plates were sterilized before use, with ultraviolet irradiation, for 2 hours. The irradiation time was determined experimentally to have a minimal effect on the cell-adhesion-related properties of the proteins while it prevented bacterial contamination. Subsequently, the wells were blocked with 200 μl/well Hank’s balanced salt solution (Sigma Chemical), containing 10 mg/ml bovine serum albumin, 50 IU/ml penicillin, and 50 μg/ml streptomycin, for 2 hours at 37°C.

In several instances, BRP were synchronized for the experiment by serum deprivation and 1.2 mM hydroxyurea, according to methods previously used for various cells, including retinal pericytes. More specifically, the culture medium was removed and, after thorough washing with Versene buffer, the cells were incubated in RPMI 1640 supplemented with 1% calf serum for 48 hours. Then the serum-deficient medium was replaced by complete growth medium containing 10% calf serum. Six hours later, concentrated hydroxyurea stock solution was added to a final concentration of 1.2 mM. After 12 hours, the cells were washed twice with RPMI 1640 and used in the proliferation assay. The BRE cells were not used after synchronization because they were very sensitive to the procedure.

To perform the assay, the cells were released with trypsin (as it was described for subculturing), washed once in the corresponding medium by centrifugation at 1000 rpm × 5 minutes, and resuspended in culture medium. Cells were then seeded onto sterile 96-well Immulon 1 plates (3,000 BRP/well and 20,000 BRE cells/well) that had been previously coated with either control or glycosylated BM macromolecule or whole BM fragments and blocked with bovine serum albumin (as previously described). Cells were allowed to adhere for 4 to 8 hours (BRP) or for 20 to 24 hours (BRE cells) on the individual macromolecules, and for 3 to 4 hours on whole BM at 37°C. At this time, the percentage of adhesion was evaluated by microscopic examination and estimated to be 50% to 80% of the cells plated. Nonadherent or nonviable cells were removed by washing twice with 100 μl/well Versene buffer. Culture medium (200 μl/well) was added and the cells were allowed to grow at 37°C, in all the plates, except for one set that was examined immediately and was considered to represent the time-zero phase of the experiment (day 0). The following time points were examined subsequently every 24 or 48 hours. The plates with BRE had their medium changed every third day.

For each time point, the cells were released from their respective wells after two gentle washings with 100 μl/well Versene and treatment with 100 μl/well trypsin-EDTA for 10 minutes at 37°C. The number of cells per well was determined using an automated Coulter (Hialeah, FL) model ZM analyzer, by transferring the trypsinized cells in vials filled with 8 ml Isoton II (Fisher Scientific, Pittsburgh, PA).

The results were confirmed by three experiments for each cell-line/substrate combination examined, using in each experiment four or six wells for each
treatment of the substrate, at every time point. For
the statistical analysis, the growth curves in each experi-
ment were drawn over the means of the four- or six-
fold determinations at each time point. Then we evalu-
ated the proliferative effect of each substrate treat-
ment by comparing these growth curves in the total
number of experiments performed for each cell-line/substrate combination, using multivariate analysis of
variance (MANOVA) for repeated-measures designs, followed, when more than two treatments were compared, by Duncan’s multiple-range test to correct for multiple comparisons. The statistical analysis was performed using the GLM procedure of the statistical computer package SAS/STAT, v. 6.08, developed by the SAS Institute (Cary, NC).

RESULTS

The extent of glucose incorporation to isolated BM macromolecules, after the glycation method described
above, has been determined before at 50 mM glucose,
an average of 0.9 or 1.35 nM glucose were incor-
polated, in stable condition, per nM of laminin or
type IV collagen, respectively. At 500 mM glucose, the
corresponding numbers were 11.04 and 12.02 nM.
The protein solutions, after nonenzymatic glycosyla-
tion, had minimal spectroscopic changes, when exam-
ined for fluorogens at an excitation/emission wave-
length of 335/385 nm and at 370/440 nm. After a 3-
day-long incubation with glucose, the BM molecules
had mainly Amadori-like modifications.

The RBM, incubated for 6 to 15 days, with no sugar or 1 M ribose, was examined for fluorogens with excitation/emission maxima at 370/440 nm; that is, the spectrometric characteristics of the collagen-
linked fluorescence (AGEs), as described previously in
studies onto collagen extracted from biopsy samples,
taken from persons with diabetic complications. The
RBM incubated with ribose demonstrated 1.8 to 3.5
times higher fluorescence values than did the control
sample.

We determined the coating efficiency of BM micro-
fragments onto the plastic wells, used for the prolif-
eration assay, by plating 125I-labeled EHS-BM that had
been incubated under similar conditions. EHS was
used as a model for BMs, because it is more abundant
than other BM. The coating efficiency was similar for
both the glycated and the control samples, in a range
of concentrations between 8 and 32 µg/ml, and de-
creased linearly from 74% to 48% of the added mate-
rial, depending on the coating concentration. At a
concentration of 10 µg/ml, which was used for the
proliferation assays, in a volume of 100 µl/well, ap-
proximately 0.7 µg matrix per well was retained on
plastic. The coating efficiency of glycated and control
BM macromolecules was determined to be slightly
increased for the glycated molecules compared with
the control. However, in these experiments, the cell
numbers at time 0 (4 to 24 hours after seeding), when
the cells demonstrated adhesion equal to 50% to 80%
of the cell number added, were not significantly dif-
ferent between control and glycated substrates; the cells
were allowed to interact with the substrate for a longer
period, compared with previous, brief cell adhesion
assays. This phenomenon was also previously ob-
served by our group for human mesangial cells.

An initial experiment was designed to compare
glycation induced to BM adsorbed onto plastic with
glycation induced to matrix suspended in solution and
then coated on plastic, regarding the effects of this
glycated matrix when used as a substrate for the prolif-
eration of retinal microvascular cells. We used 0.6 µg/
well EHS-BM adsorbed onto plastic, in six wells for
each condition examined at each time point. The ma-
trix was either already glycated after coincubation with
ribose in solution (Fig. 1A), or it was modified after
coating by adding ribose solution in the wells and
incubating the plates (Fig. 1B), as described previously
in Materials and Methods. For each method of glyca-
tion, control samples of EHS-BM were prepared by
omitting ribose from the incubation solution. The
BRP were proliferating significantly more slowly on
the glycated matrix compared with control, with both
methods of glycation used (Fig. 1A, P = 0.025; Fig.
1B, P = 0.001). We chose to use matrix glycated in
solution for the following experiments because we
were able to obtain by fluorescence, specific data
about the modifications induced by this method, and
also because in solution the amino groups of the ma-
trix are better exposed to react with reducing sugars
in the environment and to create cross links with other
free amino groups.

We evaluated the growth curves of the BRP and
the BRE cells cultured on control and glycated BM
macromolecules, or whole RBM, by determining cell
numbers at various time points, every day or every
second day.

Figure 2A shows that on glycated type IV collagen
(50 mM glucose, 0.6 µg type IV collagen adsorbed
per well), synchronized bovine retinal pericytes were
proliferating more slowly than on control (unmodi-
fied) type IV collagen, already by the first day after
adhering on the substrate. The difference in the prolif-
eration rate increased until the fourth day, when
the cells on glycated type IV collagen were 33% less
than on control, and attenuated until the end of the
experiment, when the cells on glycated type IV colla-
gen were 9% less than on control. The growth curve
on glycated type IV collagen was significantly differ-
ent from control (P = 0.02). On laminin (2.5 µg adsorbed
per well), the maximum difference, which was ob-
served on the third day, was equivalent to 17% less
growth on glycated laminin (50 mM glucose) than on
control (Fig. 2B). However, the growth curves were
Matrix Glycation

FIGURE 1. Growth curves of bovine retinal pericytes cultured on control (−−−−−) and diabetically modified (---•---) Engelbreth-Holm-Swarm tumor basement membrane (EHS-BM). The EHS-BM had been glycated (A) during incubation with ribose in solution, and then was coated on the plastic wells, or (B) it had been coated on plastic and then modified by adding a ribose solution in the wells. The proliferation rate, which equals the number of cells at a given time, divided by the number of cells that adhered initially, is plotted against the duration of each experiment in days. Each point represents the mean ± standard deviation in six determinations. The growth curve on glycated EHS-BM differed significantly from control (A: P = 0.025, B: P = 0.0001) according to multivariate analysis of variance for repeated measures.

The growth curves of the BRP on type IV collagen or laminin modified by 500 mM glucose were similar to those obtained after seeding these cells on macromolecules modified by 50 mM glucose. In addition, nonsynchronized retinal pericytes, used in proliferation assays, gave similar results (data not shown).

The BRP were also proliferating significantly more slowly (by 20% to 30%) on glycated, whole RBM compared with control RBM (Fig. 2C) following a pattern similar with that on type IV collagen. In Figure 2C, each point represents the mean ± standard deviation of cell growth in two experiments (four or six replicate determinations in each experiment), with 0.7 μg RBM adsorbed per well. A third experiment with 2 μg RBM...
adsorbed per well yielded a similar difference between the two proliferation curves, although the greater amount of matrix produced significantly faster proliferation rates. The growth curve of the retinal pericytes on glycated RBM was significantly different from control \((P = 0.04)\). Experiments on glycated and control EHS produced similar results for the pericytes (Fig. 1A).

The bovine retinal endothelial cells had a different growth response from BRP on control and glycated BM substrates. Type IV collagen (2.5 \(\mu g\) adsorbed per well), even when modified by 500 mM glucose, caused a short-term change in the proliferation rate of BRE: a 16\% increase in the first and second day after adhesion (Fig. 3A). The difference between the growth curves on control and glycated substrate was not significant. On the contrary, laminin (4.5 \(\mu g\) adsorbed per well), modified by 50 mM glucose, induced a consistently faster growth rate of BRE \((P = 0.025)\) compared with the growth of these cells on control laminin (Fig. 3B). A maximum increase of 25\% was observed on the third day of the experiment, and until the sixth day the BRE on glycated laminin were 18\% more than on control. Each point of the growth curves in Figures 2A and 2B represents the mean ± standard deviation of cell growth in three experiments (four or six replicate determinations in each experiment, for each point).

BRE cells also had a faster proliferation rate on glycated RBM (0.7 \(\mu g\) adsorbed per well), compared with that on control RBM, and the difference (25\% to 30\% increase) was not overcome until the end of the experiment (Fig. 3C: significant difference between the growth curves, \(P = 0.01)\), which was similar to the case of BRE on laminin. Each point of the growth curves in Figure 3C represents the mean ± standard deviation of cell growth in three experiments (four or six replicate determinations, in each experiment for each point). However, in experiments using glycated or control EHS-BM as a substrate, we observed no significant difference on the proliferation rate of BRE cells (data not shown).

**DISCUSSION**

The proliferation rate of retinal pericytes, when seeded on glycated type IV collagen, decreased significantly, reaching a maximum decline of 33\% of the control on the fourth day of the experiment; this decrease was not overcome completely until the seventh day in culture. On glycated laminin, the growth rate of BRP showed only a brief, insignificant decline. A significant decrease of the proliferation rate of BRP was also observed, after seeding the cells on glycated BM-like, EHS tumor matrix (which contains type IV collagen in abundance), as well as on glycated whole RBM (by 20\% to 30\%) when compared with the control.

The growth rate of retinal endothelial cells increased by 16\% to 25\% on glycated laminin compared with the corresponding growth rate of the control: a
significant increase of the growth curve throughout the experiment. On glycated type IV collagen, an increase of 16% was noted but overcome within 4 days after starting the experiment: The growth curves were deemed not to differ significantly.

When BRE cells were seeded on control or glycated EHS, no significant change of their growth rate was observed. However, when glycated retinal BM was used as a substrate, an increased proliferation rate (by 25% to 30%) was observed compared with control RBM. The differential response on these two matrices may be due to some component that is used preferentially by retinal endothelial cells, and which may not be present in the isolated EHS. Structural data about the organization of RBM33 support the hypothesis that retinal endothelial cells could interact preferably with laminin, as laminin is distributed closer to the retinal endothelial cells surface, whereas type IV collagen is distributed throughout all the BM layers, surrounding the pericytes. During the preparation of EHS-BM from lathyritic EHS tumor, most of the laminin may have been extracted by EDTA,34 because the EHS tumor is not extensively cross linked. On the contrary, the RBM is more cross linked and may be less susceptible to extraction of individual components during the isolation protocol, when no EDTA has been used. Type IV collagen is an abundant and stable component of EHS-BM and RBM; this may be the reason that both matrices, when glycated, caused decreased proliferation of BRP, the same effect that glycated type IV collagen caused to BRP.

The two kinds of cells from the retinal microvasculature responded to glycated substrates differently, as shown by the proliferation rate. The retinal pericytes demonstrated a decreased growth rate, which could be correlated with the pericyte dropout observed typically and early in retinas affected by diabetic retinopathy.35 Studies of retinas of mice36 and rats,37 after in vivo [3H]-thymidine incorporation, showed that these cells have a very low proliferation rate, under normal or diabetic conditions. However, this detection method is not very sensitive because it detects cycling cells solely through the narrow window of the S phase;37 Gordon et al,36 comparing in vivo thymidine labeling with detection of cyclin/proliferating cell nuclear antigen, found a fivefold greater sensitivity of the latter method in detecting replicating cells in rat tissues. Therefore, the pericytes in vivo can probably proliferate; even with a long turnover time, throughout the years of hyperglycemia preceding the development of diabetic retinopathy, a diabetes-induced decrease of replication could contribute to pericyte loss because of impaired cell renewal,39 especially given an increased rate of cell death. The inhibiting messages from the glycated substrate to the retinal pericyte proliferation, observed in vitro in the present study, could also create an insufficient environment for maintenance in vivo. This hypothesis calls for further investigation.

We observed an increase of endothelial cell proliferation on glycated substrate, especially glycated laminin or whole RBM. Nonenzymatic glycosylation of proteins occurs in any period of hyperglycemia during diabetes, but their accumulation depends on the duration and consistency of hyperglycemia, the turnover rate of the protein “host,” and also the local defending and scavenging ability.39 Different proteins could be accumulated in glycated form at different times, and type IV collagen, due to its extensive cross linking, is likely to be accumulated in a glycated form in less time than is laminin. Therefore, effects of glycated type IV collagen (i.e., decreased pericyte proliferation, according to our experiments) could appear, pathologically, sooner than the effects of glycated laminin (i.e., increased endothelial cell proliferation within the neovascularization process of the proliferative diabetic retinopathy).35 Proliferative diabetic retinopathy has been considered by researchers as a result of growth factor activity40 and AGEs can prompt release of growth factors.41 In addition, laminin has growth factor domains and activity in its molecular structure, and conformational modifications through glycation could influence the phenotype of adjacent cells toward proliferation, as we observed in the present study. Growth factors and biologically active extracellular matrix proteins form an integrated signaling system,43 which could have a profound role in the pathogenesis of diabetic retinopathy at the cellular level.

Type IV collagen and laminin were glycated by a short incubation of 3 days under high glucose concentrations. This short period was selected to preserve the molecules intact and to minimize aggregation. At the end of this incubation, at least one molecule of glucose was bound nonenzymatically onto each macro-molecule.20 The modifications induced to the BM molecules probably were mainly Amadori-like, but we cannot exclude the presence of AGEs. The fluorescence method is not sensitive enough compared with the use of anti-AGE antibodies, according to previous reports.44,45

We determined the fluorescence values (excitation 370 nm/emission 440 nm) of the glycated matrix we used: The glycated RBM had fluorescence values 1.8 to 3.5 times greater than the control samples. Many researchers have reported data about fluorescent AGEs in diabetic tissues. Monnier et al13 showed that digested skin collagen from persons with insulin-dependent diabetes mellitus had fluorescence values twice as high as did age-matched control subjects. Moreover, the severity of retinopathy in the patients was reported to increase linearly with the fluorescence level.15 Collagen-linked fluorescence, in streptozocin-induced diabetic rats, was also measured in the renal...
cortex (≈1.5 times higher than in controls) by Mitsuhashi et al., and in unstained preparations of retinal vessels (2.6 times the corresponding value in normal age-matched animals) by Hammes et al. In addition, quantitation of the cells of the retinal microvasculature, in microscopic sections of the rat retina, indicated that diabetes increases the retinal endothelial cell number by 23% and decreases the pericyte number by 30%.

Thus the fluorescence values of the in vitro glycated BM, used in our experiments, were similar to the corresponding values of the retinal BM in persons or animals with diabetic retinopathy. This in vitro glycated, whole BM and individual BM components induced changes to the cell numbers in culture that were comparable to the changes observed in diabetic animal retinas in situ. In addition, and in support of our observations, aminoguanidine, which inhibits AGE formation, was found to prevent the diabetes-induced proliferation of endothelial cells and reduce the magnitude of pericyte loss in diabetic rats.

The mechanisms that cause the differential proliferation rate of the retinal microvascular cells, in response to glycated BM macromolecules and RBM, must still be elucidated. Retinal microvascular cells may interact differently with the glycated substrate due to a conformational alteration or glycation of a specific cell-adhesive recognition site. This hypothesis could explain the absence of dose dependency when the retinal microvascular cells interact similarly with BM macromolecules altered under 50 or 500 mM glucose, as well as the similarity of the effects produced by the Amadori-modified molecules and by the AGE-glycated matrix. Alternatively, these cells may have specific receptors for AGE or Amadori products that recognize specifically the glycated BM. Receptors for AGE products were previously described in macrophages, glomerular mesangial cells, and aortic endothelial cells; receptors for Amadori-products have been described in aortic endothelial cells.

Difference in the receptors and/or the intracellular messages developing after interaction with the glycated substrate could cause the observed different response from the two kinds of retinal microvascular cells: decreased growth rate of BRP and increased growth rate of BRE cells. Whatever the mechanism, the obtained data indicate that in vitro glycation of BM could result in an altered growth rate of retinal microvascular cells in culture. Still to be determined is whether glycation-related matrix changes are involved in the development of diabetic retinopathy in situ.

**Key Words**

diabetes, extracellular matrix, nonenzymatic glycosylation, retinal microvascular cells, retinopathy

**Acknowledgments**
The authors thank Jennifer D. Thull for assistance in preliminary experiments and Howard Higson for technical support in the laboratory. They also thank Stefanos Manganaris (Computer Science Department, Vanderbilt University) for assistance with the statistical analysis and use of the SAS package.

**References**

Matrix Glycation


