High Glucose Inhibits Retinal Capillary Pericyte Contractility In Vitro

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Purpose. To study the effect of high glucose concentrations on pericyte contractility.

Methods. Bovine retinal capillary pericytes were cultured on silicone rubber sheets, which could be seen to wrinkle when a cell contracted. Cells were grown in glucose, or mannitol, in concentrations ranging from 5 to 40 mMol. Pericyte contractility was expressed as the percentage of cells wrinkling the silicone substratum. Observations were made fortnightly for 8 weeks.

Results. Cells grown in glucose exhibited a dose-dependent inhibition of contractility that was significantly greater than that seen with cells grown in mannitol, which were affected to a lesser extent. After returning to normoglycemic conditions for a further 4 weeks, the contractility of cells grown in lower glucose concentrations recovered partially, but cells grown in 40 mMol glucose did not recover at all. Pericyte proliferation was also impaired by the high-glucose growth medium.

Conclusions. Pericyte contractility is inhibited by high glucose concentrations. This is consistent with the hypothesis that increased retinal blood flow may be a factor in the early pathogenesis of diabetic retinopathy. Invest Ophthalmol Vis Sci. 1993;34:3396-3401.

More information is needed about the early cellular changes in the pathophysiology of diabetic retinopathy because it remains poorly understood.1

Derangement of pericyte function may well be an important factor. Pericyte dropout, a feature of diabetic retinopathy,2,3 may result in endothelial cell proliferation due to loss of contact inhibition.4 The presence of aldose reductase in pericytes5,6 provides a mechanism that may explain pericyte dysfunction.

The ability of pericytes to contract has been demonstrated in vitro, using silicone rubber and collagen lattice assays,2 and in vivo.8,9 Pericytes contain several contractile proteins including muscle actins,10-12 of which muscle and nonmuscle isoforms have been identified,13 tropomyosin14 and isomyosins,15 as well as cyclic guanosine monophosphate-dependent protein kinase,16 which is thought to regulate smooth muscle contraction. Pericytes have been shown in vitro to be electrically excitable cells,17 and to contract in response to endothelium-derived vasoactive factors.18 These observations have led to speculation that one of the major functions of pericytes might be to regulate microvascular blood flow, although some investigators suggest that the main effect of pericyte contraction is to regulate endothelial permeability.9,19

We hypothesized that high glucose might impair pericyte contractility, thereby encouraging the development of diabetic retinopathy through a disturbance of retinal vascular autoregulation or permeability. In this study we assayed the contractility of bovine retinal capillary pericytes grown in normoglycemic, hyperglycemic, and hyperosmolar but normoglycemic media, without added vasoconstrictive factors, by growing them on silicone rubber substrata. Our data suggest that a hyperosmolar environment impairs pericyte contractility, but more so in the presence of high glucose. We also provide data that confirm that high glucose inhibits pericyte proliferation.

METHODS

The study adhered to the guidelines set out in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research (May 1992).

Cell Culture

Bovine retinal capillary pericytes were isolated using previously described techniques.20,21 Briefly, three bo-
vine retinas were removed and rinsed three times in Eagle’s minimal essential medium for 90 minutes. Then they were cut into small pieces and vortexed in 15 ml of enzyme mixture (500 μg/ml collagenase type I [Sigma Chemical Co., St. Louis, MO], 200 μg/ml DNAse [Sigma] and 200 μg/ml pronase [Boehringer Mannheim, GmbH, Mannheim, Germany]) for 5 seconds then digested in a shaking water bath for 20 minutes at 37°C. Microvessel fragments trapped on a 53-μm nylon mesh were centrifuged at 400g for 7 minutes. The pellet was resuspended in Dulbecco’s modified Eagle’s medium with 5 mMol D-glucose and 20% fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml Amphotericin B (Squibb, Princeton, NJ) in tissue culture flasks and cultured at 37°C with 5% CO₂. Medium was changed after 24 hours then twice-weekly. Cells were passaged after 10 days in primary culture with 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid.

Second passage cells were divided into five groups and grown in media with 6, 20, and 40 mMol D-glucose (groups I, III, V, respectively), 5 mMol D-glucose and 15 mMol mannitol (group II), 5 mMol D-glucose and 35 mMol mannitol (group IV) with 150,000 cells per 25 cm² flask. A concentration of 20 mMol glucose was chosen, which is commonly found in persons with diabetes, whereas 40 mMol glucose was chosen as a high level, which can occur in persons with diabetes, but which might also be expected to magnify subtle effects. After observation in these conditions for 8 weeks, the cells were returned to the normoglycemic environment used for the primary culture. All experiments were performed in quadruplicate and repeated twice.

To keep the cell density of the five groups as uniform as possible, cells were passaged at the end of each week, counted with a hemocytometer and replated at a fixed density of 150,000 cells per 25 cm² flask. Because of this requirement for frequent passing, cell proliferation was expressed as n₁/n₀ where n₁ = cell number per flask 1 week after passaging and n₀ = the initial cell number when the cells were passaged (150,000).

**Cell Contractility**

Silicone-treated coverslips were prepared according to a previously described technique. Brieﬂy, this entailed spreading Dimethylpolysiloxane (60,000 centistokes; Sigma) thinly on 22 × 40 mm glass coverslips and exposing it to the middle part of the bunsen burner flame for approximately 2 seconds. Under-heating of the silicone resulted in poor cell attachment, whereas overheating caused wrinkles in the silicone before the cells attached. These coverslips were affixed with a little fresh silicone to the bottom of 60-mm tissue culture dishes which had 2 × 2 mm grids inscribed on their bases.

Pericytes were transferred into these tissue culture dishes (75,000 cells per dish) for the contractility assay after 1, 3, 6, and 8 weeks in the hyperosmolar environment, then 2 and 4 weeks after their return to normoglycemia.

After 24 hours of growth on silicone sheets, cells from ten randomly selected 2 × 2 mm squares were examined by a single masked observer. The proportion of cells that could be seen to wrinkle the silicone film was expressed as the percentage of the total number of cells present. A cell was scored as contracting if two or more wrinkles could be seen, or if one wrinkle was longer than the transverse diameter of the cell. Initially, cells were checked for viability with trypan blue until it became evident that all attached cells excluded the dye, with dead cells rounding up and detaching into the medium.

**Alpha Smooth Muscle Actin Staining**

Pericytes were stained for alpha smooth muscle actin after 3 and 8 weeks in culture using an avidin biotin kit (Sigma), in which the primary antibody was mouse monoclonal anti-α-smooth muscle actin and the secondary antibody was biotinylated goat anti-mouse immunoglobulin.

**Statistics**

Results are expressed as mean ± SD, and compared using the unpaired Students’ t test. Results quoted as significant afforded a P value of 0.05 or less. The comparisons of most interest, which were identified prospectively, were between groups I, IV, and V. Some of the error bars in the graphs are not evident because they are smaller than the plot symbol.

**RESULTS**

**High Glucose Inhibited Pericyte Contractility (Fig. 1)**

After culture for 1 week in their respective media, less cells from the hyperosmolar and high-glucose groups (groups II, III, IV, and V) exhibited an ability to contract compared with cells from the normoglycemic group (group I) (group I 91.8 ± 3.2%, group II 70.2 ± 1.0%, group III 71.3 ± 3.2%, group IV 71.9 ± 1.9%, group V 62.8 ± 2.9%). Although groups II, III, and IV demonstrated a similar impairment, cells grown in 40 mMol glucose (group V) were significantly more affected compared with cells grown in 35 mMol mannitol and 5 mMol glucose (group IV; P = 0.003).

This pattern was maintained when the cells were further examined after 3, 6, and 8 weeks in culture. After 8 weeks in culture, although no significant dif-
High Glucose Inhibited Pericyte Proliferation

The amount of cell proliferation at the end of each passage declined sharply in all groups as the experiment progressed, but this was more marked in groups II, III, IV, and V compared with group I (Fig. 4). After 1 week in culture, the cell density had increased most in cells from group I (group I 10.6 ± 0.6, group II 9.7 ± 0.5, group III 9.6 ± 0.7, group IV 9.3 ± 0.5, group V 9.1 ± 0.4). Less proliferation was found in cells from group V compared with group I ($P = 0.004$) but not when group V was compared with group IV ($P = 0.50$). The proliferation of cells from group III did not differ significantly with that of cells from group I ($P = 0.08$) or II ($P = 0.95$). A similar pattern was seen after 8 weeks in culture (group I 4.3 ± 0.5, group II 3.3 ± 0.2, group III 3.2 ± 0.2, group IV 4.0 ± 0.1, group V 2.4 ± 0.1) although cells from group IV showed a growth spurt during the last 2 weeks of the experiment. Groups III and V showed significantly less proliferation com-
DISCUSSION

This study shows that the ability of bovine retinal capillary pericytes to contract is impaired in a dose-dependent manner when they are cultured in high-glucose concentrations. Hyperosmolar, normoglycemic conditions impaired pericyte contractility to a lesser extent. The inhibition of contractility was seen after 1 week in culture and declined slightly thereafter. Little recovery of contractile ability was found 4 weeks after cells were returned to normal glucose levels. The proliferation of pericytes was also inhibited by high glucose concentrations.

The question arises as to what the silicone wrinkling assay actually measures. We attempted to measure the size and number of wrinkles present, but we found that the simple presence or absence of wrinkles was by far the most quantifiable and reproducible observation. Cells that did not wrinkle the plastic were still viable in that they remained attached to the substrate and excluded the passage of trypan blue. It is likely that a critical tension is required to form a wrinkle and some cells were unable to achieve this, in which case the assay might well reflect cellular contraction. Conversely, part of the wrinkling process requires cel-

FIGURE 4. Proliferation (n1/n0) of cells from groups I (open circles), II (open squares), III (closed circles), IV (closed squares) and V (triangles) assessed weekly during 8 weeks of culture in their respective media, where n1 = cell number per dish 1 week after passaging and n0 = cell number per dish when passaged (150,000). Coordinates are displaced horizontally to facilitate the assessment of error bars.

pared with group I at 8 weeks (group I vs group III, \( P = 0.01 \), group I vs group V, \( P = 0.001 \)). Although group II did not differ significantly from group III at 8 weeks (\( P = 0.80 \)), group V cells showed significantly less proliferation compared with group IV cells from week 2 onward (e.g., at 8 weeks, \( P < 0.001 \), at 6 weeks, \( 1.8 \pm 0.1 \) vs \( 2.2 \pm 0.1 \), \( P = 0.002 \)).

Morphology

All groups showed greater than 95% positive staining for anti-alpha-smooth muscle actin after 3 and 8 weeks in culture. Group I cells showed darker staining and more regular orientation of actin filaments compared with cells from group V (Fig. 5). All cells from groups I, II, and III, and most from groups IV and V, displayed typical pericyte morphology with large cell bodies and elongated, spindly processes. Some cells from groups IV and V displayed degenerative features, including irregular size tending toward enlargement, but nevertheless they excluded trypan blue while they were attached to the silicone.

FIGURE 5. Anti-alpha-smooth muscle actin of cells grown for 8 weeks in (A) normoglycemic medium and (B) 40 mMol glucose.
ular attachment to the substratum, which might also have been affected by high glucose. This latter mechanism might have the same functional implications in vivo as does the former.

The difference between the high glucose and the hyperosmolar, normoglycemic groups was only seen when cells grown in 40 mMol glucose were compared with those grown in 35 mMol mannitol and 5 mMol glucose. Longer exposure of pericytes to more moderate glucose concentrations might show more separation between high-glucose and hyperosmolar environments, but this is not possible in vitro. Lorenzi found a deleterious effect of high mannitol concentrations on endothelial cell replication when compared with the effect of high glucose, also to a lesser extent. 

Hyperosmolality of its own may have various adverse effects on cellular homeostasis, including a disturbance of myoinositol metabolism.

A number of possible explanations for the inhibition of pericyte contractility by high glucose concentrations may be proposed. High glucose levels cause increased activity of the aldose reductase pathway, which leads to increased intracellular sorbitol levels, depletion of intracellular myoinositol, and impairment of Na\(^+\)-K\(^+\) adenosine triphosphatase activity. Decreased Na\(^+\)-K\(^+\) adenosine triphosphatase activity in vascular smooth muscle is a potential cause of rearrangements in the regulation of vascular tone and of altered responses to vasoactive factors. 

1,2-diacyl-sn-glycerol accumulation and protein kinase C activation, which also result from increased activity of the sorbitol pathway, are associated with increased vascular clearance of albumin and blood flow in rat granulation tissue. Conversely, nonenzymatic glycosylation, which occurs over weeks to years, might affect cellular proteins, especially, perhaps, contractile proteins or enzymes, or factors in the media that initiate contraction.

The temporal characteristics of the inhibition in this study may indicate the nature of the underlying mechanism. Inhibition of contractility was seen when the cells were first examined after 1 week in culture. Thereafter the amount of inhibition increased only slightly. This suggests a mechanism involving increased activity of the aldose reductase pathway rather than nonenzymatic glycosylation. When the cells were grown for a further 4 weeks in normoglycemic conditions, however, their contractility did not recover. This is in keeping with a mechanism involving nonenzymatic glycosylation of cellular proteins. It is possible that both processes play a role.

The inhibition of pericyte proliferation by high glucose concentrations confirms a previous report. Little information is available about the turnover and replicative potential of pericytes in vivo. However, the inhibition of pericyte proliferation by high glucose seen in vitro may explain the disappearance of pericytes found in diabetic retinopathy.

The ability of high glucose levels to directly and rapidly impair retinal pericyte contractility in vitro is consistent with studies on diabetic retinopathy in animals and humans. Impaired pericyte contractility may lead to capillary dilatation, which is one of the earliest manifestations of diabetic retinopathy in rats and humans. This may explain the increased retinal blood flow that was found in patients with background, preproliferative and proliferative diabetic retinopathy when compared with flow in nondiabetics and diabetics without retinopathy. Hyperperfusion, combined with increased blood viscosity and decreased red cell deformability, which also occurs in diabetes, may damage retinal capillary endothelial cells through increased shear stress, leading to capillary closure, retinal ischemia, and their sequelae.

**Key Words**

pericyte, hyperglycemia, contractility, retina, proliferation

**Acknowledgments**

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**References**

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