Human Retinal Vascular Cells Differ from Umbilical Cells in Synthetic Functions and Their Response to Glucose (43345)

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Abstract. **Cell culture systems have commonly been used to study mechanisms implicated in the pathogenesis of diabetic retinopathy, but the great majority of cell preparations used have been either of nonhuman retinal origin or nonretinal human origin. Because of questions of species and organ specificity in the function of cells of vascular origin, in this study, cultured microvascular endothelial cells (HREC), pericytes (HRPC), and pigment epithelial cells from the postmortem human retina, and endothelial cells from human umbilical vein (HUVEC) were evaluated with respect to cell proliferation, and secretory products potentially important in diabetic retinopathy, i.e., prostaglandins (PG) and plasminogen activators (PA), normalized to DNA content/well, under both basal (5 m*M*) and high (25 m*M*) glucose conditions. Glucose (25 m*M*) reduced DNA content similarly in both types of endothelial cells, had a lesser effect on HRPC, and did not significantly alter the proliferation of pigment epithelial cells. Basal secretion of PGI_2 (measured as 6-keto-$PGF_{1\alpha}$) was in the order HRPC ≫ HREC > HUVEC, whereas PGE_2 secretion was in the order HREC ≫ HRPC > HUVEC. Glucose (25 m*M*) stimulated PGI_2 secretion by HRPC, but not by either type of endothelial cell, and enhanced PGE_2 secretion by HREC, but not by HUVEC or HRPC. Release of plasminogen activator activity differed between HUVEC and HREC under basal conditions and addition of 25 m*M* glucose stimulated release only from HREC. Glucose (25 m*M*) stimulated PA secretion by HREC, but not by HUVEC. These findings provide evidence that human retinal pericytes are an important source of prostacyclin, and that there are differences between HREC and HUVEC with respect to secretory functions and their modulation by glucose, indicating regional specificity of these functions. Extrapolation to human retinal vascular cells from experiments using cells from heterologous vascular beds to draw inferences about the pathophysiology of diabetic retinopathy are not valid for these cellular functions.** [P.S.E.B.M. 1992, Vol 199]

The pathogenesis of proliferative diabetic retinopathy remains poorly understood, particularly the contributions of the various cellular constituents of the retina early in this disease process. Most experimental models previously available to study these cellular elements in isolation have been derived from either nonhuman retinal tissue or human vessels of nonretinal origin. Because vascular cells display species and organ specificity and are morphologically and functionally heterogeneous (1–5), we have established microvascular endothelial cells and pericytes, as well as pigment epithelial cells, from the normal postmortem human retina in culture. The present studies were designed to: (i) evaluate the ability of endothelial cells and pericytes from human retinal microvessels to secrete products likely to be important early in the development of diabetic retinopathy; (ii) determine the responses of those functions and cellular proliferation to a pathologic glucose concentration; and (iii) compare the responses with those found with a commonly used human endothelial cell preparation derived from a larger vessel, the umbilical vein, and determine whether the latter may serve as a model for these functions of human

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retinal microvascular endothelial cells (HREC). The functions evaluated included cell proliferation and the production of prostaglandins and plasminogen activators. Alterations in proliferation-cell survival that occur in proliferative diabetic retinopathy result in first pericyte loss, then acellular capillaries, and, finally, neovascularization (6–7). Arachidonic acid metabolites such as prostacyclin (prostaglandin [PG] I_2) modulate microvascular blood flow and permeability and function as potent vasodilators and inhibitors of platelet adherence to endothelium (1, 8–12). Hence, they are candidates to explain disturbances in local blood flow early in the course of proliferative diabetic retinopathy. The prostaglandin E series compounds both stimulate smooth muscle contractility and have complex effects on intermediary metabolism (13–18). Differences in their role between large and small vessels are just now being defined (1, 19). Plasmin generated by the tissue fibrinolytic system is not only the primary effector enzyme in fibrinolysis, but also one of the major contributors of proteolytic activity necessary for basement membrane degradation, an important initiating event in endothelial cell migration and proliferation leading to neovascularization (20–23).

Materials and Methods

Cell Isolation and Culture. Postmortem human eyes were obtained from the Cincinnati Eye Bank and the National Disease Research Interchange (Philadelphia, PA). Retinal vascular cells were isolated from a 37-year-old male donor, and pigment epithelial cells were from a 2-year-old female donor. Both donors were previously healthy victims of trauma. The eyes were opened under aseptic conditions at the ora serrata, and the neurovascular retina was mechanically separated from the underlying pigment epithelium. Following extensive washing and vigorous pipetting, microvessels were isolated by sequential sieving through nylon mesh (Tetko, Inc., Elmsford, NY), first 200 μm and then 50 μm in pore size. Fragments of microvessels retained on the latter (50 μm) nylon mesh were collected by several washes of mesh with medium M199 (Gibco, Grand Island, NY) containing 1% bovine serum albumin, followed by centrifugation (500 *g*), and then subjected to digestion with 0.01% collagenase (CLSPA; Worthington, Freehold, NJ) in M199 containing 0.5% bovine serum albumin for 1 hr at 37°C, with gentle agitation every 15 min. Digests of capillaries were suspended in growth medium (consisting of medium M199, 20% fetal calf serum (FCS; Gibco), 20 μg/ml of endothelial cell growth supplement (Sigma, St. Louis, MO), 90 μg/ml of heparin, and 2 m*M* L-glutamine) and then plated on 0.5% gelatin-fibronectin (1 $\mu g/cm^2$)-coated dishes (24). Cellular outgrowth from cultured microvessels consisted of colonies of HREC, retinal pericytes (RPC), or a mixture of both.

HREC. HREC (Fig. 1A) were isolated on a fluorescence-activated cell sorter (Becton Dickinson model 440, University of Cincinnati Flow Cytometry Lab) after labeling of cells with acetylated low density lipoprotein labeled with 1,1′-dioctadecyl-1-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (DiI-Ac-LDL; Biomedical Technologies, Stoughton, MA). This resulted in preparations in which >95% of cells showed staining not only with DiI-Ac-LDL, but also with a second marker, antibody to coagulation factor VIII (not shown).

Human retinal pericytes. Human retinal pericytes (HRPC) (Fig. 1B) were identified on the basis of morphology and failure to stain with antifactor VIII antibody or DiI-Ac-LDL. Selective growth of these cells was achieved by plating retinal microvessel fragments on uncoated plastic culture dishes in medium M199 supplemented with 10% FCS.

Human retinal pigment epithelial cells. The pigment epithelium was subjected to limited collagenase digestion and then isolated cells were plated at a density of 10,000 cells/cm^2 on uncoated dishes in minimal essential medium containing 10% FCS (25). By light microscopy at low cell density, these cells had a polygonal and often elongated morphology, whereas at high cell density (Fig. 1C), most appeared ovoid. Pigment granules were evident during early passages; however, consistent with other data concerning human retinal pigment epithelial (HRPE) cells (25), there was a progressive loss of pigment granules over time in culture.

Human umbilical vein endothelial cells. Human umbilical vein endothelial cells (HUVEC) were obtained by collagenase digestion (26) and maintained in the same growth medium as used for HREC (Fig. 1D). Greater than 95% of these cells showed immunofluorescent staining with antifactor VIII antibody and DiI-Ac-LDL (not shown).

Experimental Procedures. Both cell proliferation and cell secretory functions were evaluated after 9 days of exposure to normal (5 m*M*) or high (25 m*M*) glucose in the culture medium. HREC and HUVEC were maintained in endothelial growth medium (without heparin), and HRPE and HRPC were maintained in medium M199 supplemented with 10% FCS. All cells were plated in medium containing 5 m*M* glucose for the initial 24 hr to ensure adherence to the solid phase, and then the medium was replaced with medium containing the appropriate test glucose concentration; culture medium was subsequently replaced every 3 days. For studies of secretory function, at the conclusion of the 9-day period, the culture medium was removed and the cell monolayers were washed twice with medium M199 (warmed to 37°C) and incubated in fresh serum-free medium M199 containing 5 or 25 m*M* glucose for 2 and 20 hr, after which the medium was collected for assay. The cells were then dissociated using 0.25%

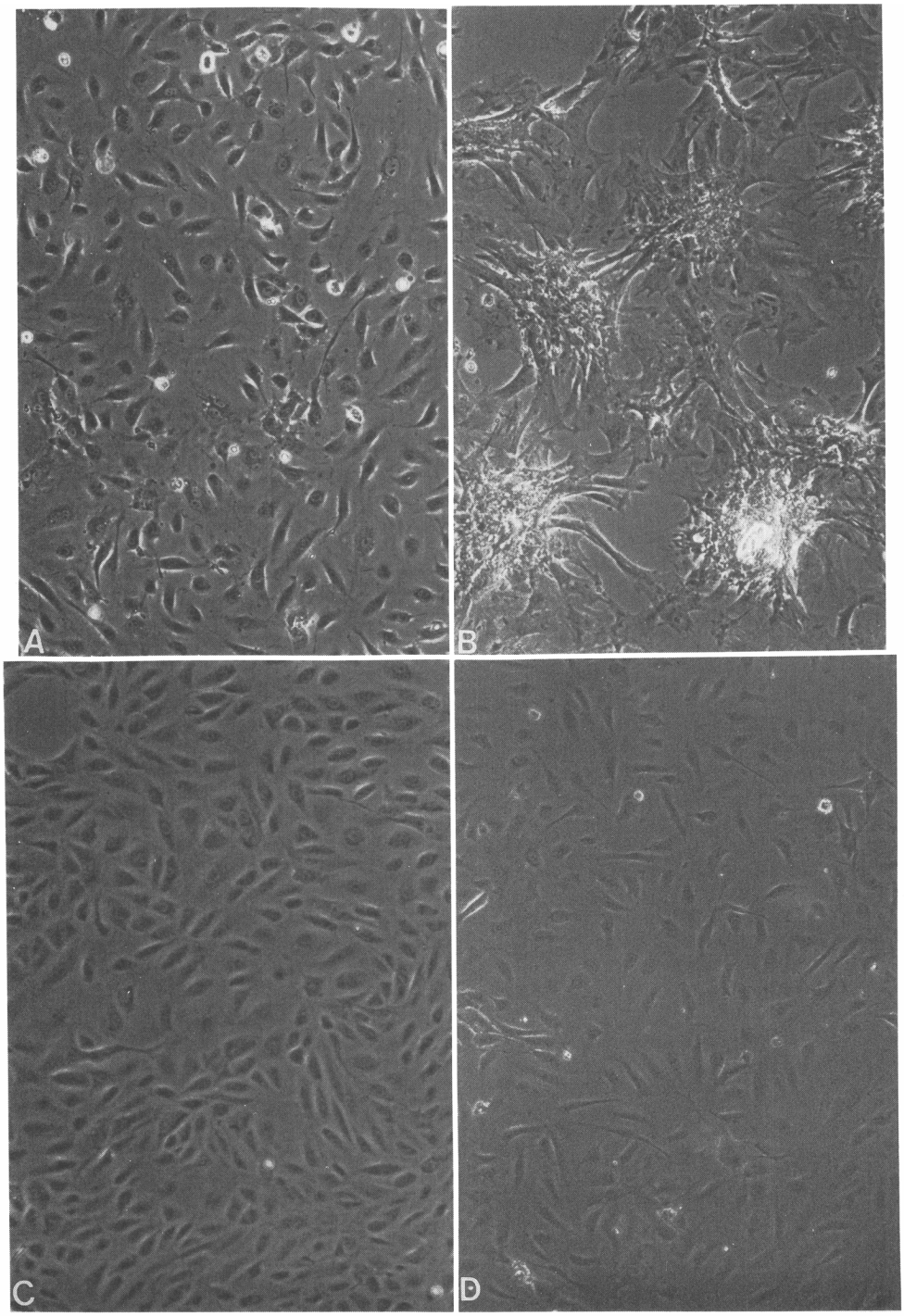

Figure 1. Phase contrast photomicrographs of human retinal cells and umbilical vein endothelial cells maintained for 9 days in 5 m*M* glucose (original magnification ×530). (A) Microvascular endothelial cells (HREC), sixth passage. (B) Microvascular pericytes (HRPC), fifth passage. (C) Pigment epithelial cells (HRPE), fourth passage. (D) Umbilical vein endothelial cells (HUVEC), sixth passage.

trypsin (Gibco) for 2 to 3 min, counted in a hemocytometer, collected by centrifugation, washed twice with M199 (without phenol red), and solubilized in acid guanidinium thiocyanate-phenol solution (0.1 ml/10^5 cells of RNAzolB; Cinna/Biotecx, Friendswood, TX) for DNA measurement (27). For studies in endothelial cells, the serum-free medium was supplemented with 20 μg/ml of endothelial cell growth supplement and 2 m*M* L-glutamine. The 20-hr incubation resulted in detachment of many cells that may cause artifacts in quantitative results. Therefore, only incubations maintained for 2 hr were used for quantitative studies. In order to exclude an effect of cell density as a possible factor modifying secretory function, and because high

glucose concentration inhibited cell proliferation, only media from wells with similar cell numbers based preliminarily on cell counts (approximately 3–4 × 10^4 cells/cm^2) were selected for assay. Final results were expressed in relation to DNA content.

Analytic Techniques. Cell proliferation was determined using a newly modified fluorescent assay for cellular DNA content. A value of 9.2 ± 0.6 pg/cell was taken as the DNA content of these cultured human cell preparations (27).

Eicosanoid determinations. Radioimmunoassays for 6-keto-$PGF_{1\alpha}$ and PGE_2 were performed in triplicate using an equilibrium single antibody procedure with a ^{3}H-labeled antigen tracer (Dupont New England Nuclear), 16-hr incubation, and a polyethylene glycol separation (28). Blank samples consisted of culture medium. The limit of detection was ~7 pg/tube for each assay. The intraassay and interassay coefficients of variation were 10% and 11% for 6-keto $PGF_{1\alpha}$, and 10% and 16% for PGE_2, respectively.

Plasminogen activator activity. The enzymatic activity of plasminogen activator (PA) was determined by zymography using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (29, 30). The acrylamide separating gel was prepared by enriching a 17.1-ml aliquot of a 9% solution with 4 ml of human fibrinogen (10 mg/ml stock solution) and 1 ml of purified bovine plasminogen (1.1 mg/ml stock solution). The stacking gel added was prepared using 3% acrylamide not containing fibrinogen or plasminogen. The anodal and cathodal running buffers both contained 45 m*M* Tris HCl, 320 m*M* glycine, and 0.086% sodium dodecyl sulfate (pH 8.0). Samples of media and serial dilutions of urokinase-type plasminogen activator (u-PA) standard were prepared in the sample buffer (125 m*M* Tris HCl [pH 6.8], 37.5% glycerol, 0.005% bromophenol, 3.5% sodium dodecyl sulfate) and loaded on the gel in a volume of 80–100 μl. Electrophoresis was carried out at 4°C at a constant current of 40 mA/slab for 60 min followed by 65 mA/slab until the tracking dye reached the bottom of the separating gel. After electrophoresis, the gels were washed twice for 30 min in 2.5% (v/v) Triton X-100, and incubated at 37°C for 16-hr in 0.1 *M* glycine (pH 8.3), stained in 0.1% amido black solution, and destained in 10% acetic acid/15% methanol. Zones of lysis appeared as transparent areas against a uniform background of darkly stained fibrinogen and were analyzed by laser-scanning densitometry. Secretion of plasminogen activator was calculated from the curve obtained with the u-PA standards and expressed in Ploug mU/μg DNA/2 hr.

Data Analysis. All data are shown as mean ± SE. Effects of glucose on cell proliferation were evaluated by unpaired *t* test. The effects of cell type and glucose on secretory functions were determined by two-way analysis of variance, followed by Duncan's New Multiple Range Test.

Results

Cell Proliferation. In order to compare plasminogen activator release and prostaglandin production among cell types on a per cell basis following prolonged culture under conditions differing in glucose concentration, it was necessary first to determine the effects of

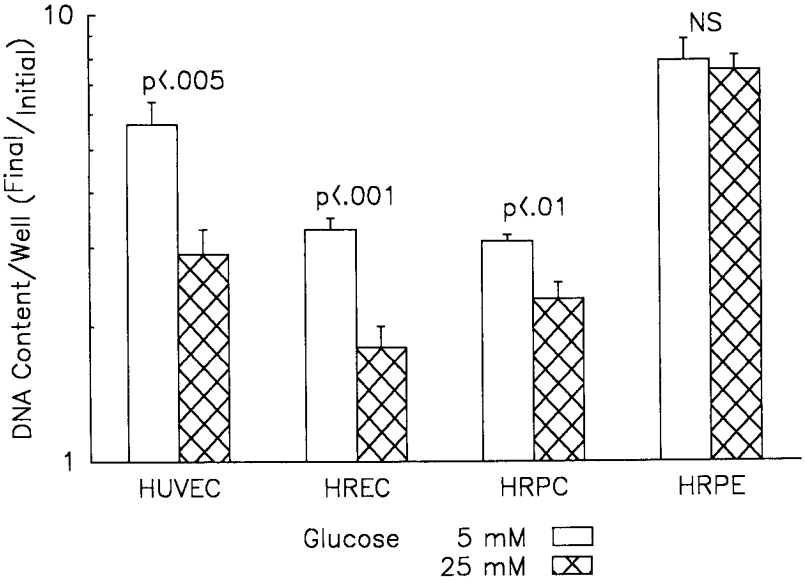

Figure 2. Effect of glucose on proliferation of cultured retinal cells derived from human postmortem eyes (HREC [*n* = 10], HRPC [*n* = 6], HRPE [*n* = 10]) and endothelial cells from human umbilical vein (*n* = 10). Determinations in endothelial cells from both origins were conducted after the fifth passage, HRPE cells after the third passage, and HRPC after the fourth passage. The ratio of DNA content per well (final to initial) shown on the ordinate is in log scale. Based on DNA content, initial plating densities were: 3.0 × 10^3/cm^2 for HREC, 4.8 × 10^3/cm^2 for HRPC, 5.0 × 10^3/cm^2 for HRPE cells, and 3.3 × 10^3/cm^2 for HUVEC.

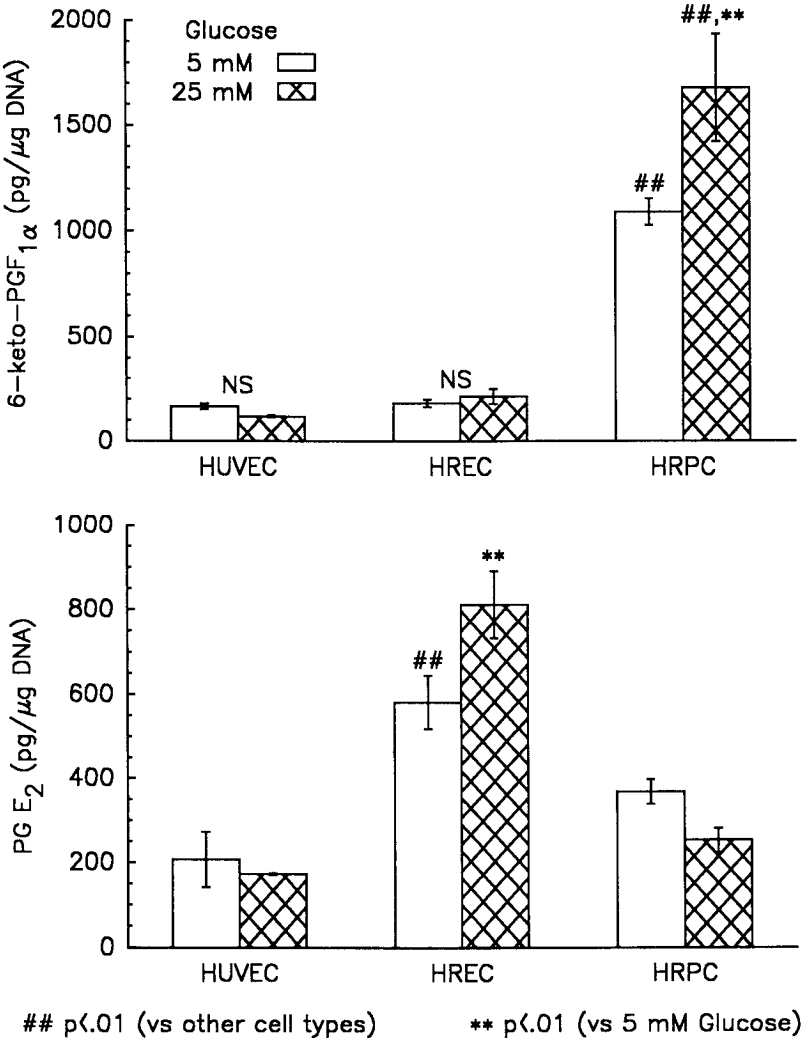

Figure 3. Prostaglandin release into culture medium: effect of glucose on endothelial cells and retinal pericytes (all measurements in triplicate). HREC and HUVEC were seeded at Passage 6 into 12-well clusters at a density of 5×10^4 cells/well and RPC (Passage 5) were plated at a density of 2.2×10^4 cells/well. Top panel, 6-keto-$PGF_{1\alpha}$; bottom panel, PGE_2.

those conditions on cell proliferation over the same interval (Fig. 2). Culture for 9 days in 25 m*M* glucose resulted in a 45 ± 6% lower DNA content for HREC and 49 ± 7% lower DNA content for HUVEC than that found at 5 m*M* glucose. Consistent with pericyte loss observed with many years of diabetes *in vivo*, the pericyte population was also reduced by high glucose, with the DNA content 24 ± 5% lower after 9 days at 25 m*M* glucose. In contrast, proliferation of HRPE was unaffected.

Prostaglandin Secretion. The content of 6-keto-$PGF_{1\alpha}$ was markedly higher in medium from pericytes than that from endothelial cells of either source (Fig. 3, top panel), in the order RPC ≫ HREC > HUVEC (1089 ± 10, 179 ± 45, 164 ± 13 pg/μg DNA/2 hr, respectively). Secretion of PGI_2 was stimulated by 25 m*M* glucose only in the cultures of pericytes and not those of endothelial cells of either origin. In contrast, basal PGE_2 secretion was less from pericytes than from HREC. There were marked differences between secretion by HREC and HUVEC, in the order HREC ≫ RPC > HUVEC (581 ± 63, 368 ± 29, 207 ± 66 pg/μg DNA/2 hr, $P < 0.01$, HREC versus HUVEC and pericytes). PGE_2 secretion (Fig. 3, bottom panel) was stimulated by 25 m*M* glucose only in the cultures of the cell type with the highest basal production, HREC (812 ± 79, $P < 0.01$), and not in cultures of either HUVEC or HRPC.

Plasminogen Activator Activity. Plasminogen activator activity found in culture medium from endothelial cells had the electrophoretic mobility of 54 kDa and 33 kDa species, consistent with the high and low molecular weight forms of urokinase-type PA. Both high and low molecular weight forms of PA were present after 20 hr of incubation (Fig. 4C), but only the high molecular weight form was present when incubation was carried out for 2 hr (Fig. 4, A, B, and D). There was no plasminogen activator activity detectable in

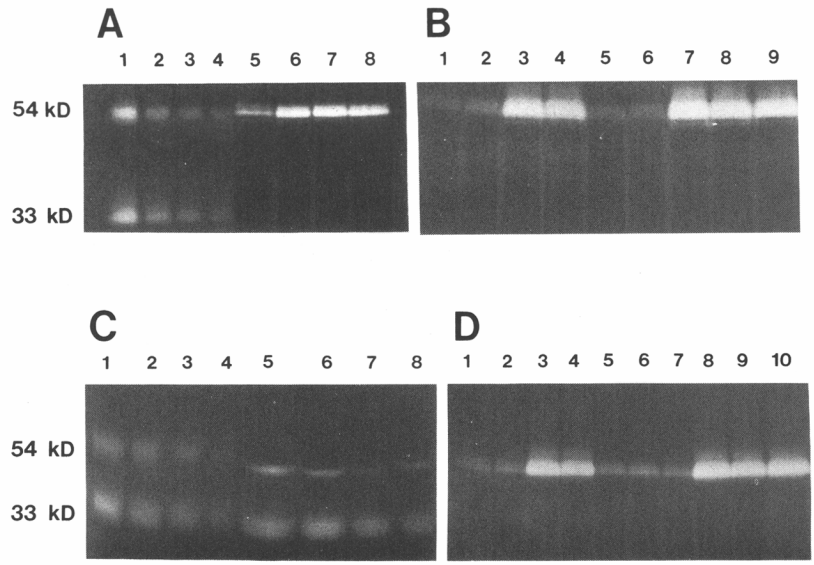

Figure 4. Representative zymograms of urokinase-type plasminogen activators in culture medium from the indicated cell preparations, maintained in 5 or 25 m*M* glucose and incubated in serum-free medium for 2 (A, B, and D) or 20 hr (C). The DNA content (shown in parentheses below) represents the quantity of cells from which the medium applied to the gel was derived. (A) Lanes 1–4 contain 30 mU, 15 mU, 7.5 mU, and 3.8 mU, respectively of both 33 kDa and 54 kDa u-PA standards. Lane 5, HREC in 5 m*M* glucose (0.18 μg of DNA); Lane 6, HREC in 25 m*M* glucose (0.18 μg of DNA); Lane 7, HUVEC in 5 m*M* glucose (0.16 μg of DNA); Lane 8, HUVEC in 25 m*M* glucose (0.15 μg of DNA). (B) HREC in 5 m*M* glucose: Lane 1 (0.16 μg of DNA); Lane 2 (0.20 μg of DNA); Lane 5 (0.18 μg of DNA); Lane 6 (0.16 μg of DNA). HREC in 25 m*M* glucose: Lane 3 (0.22 μg of DNA); Lane 4 (0.25 μg of DNA); Lane 7 (0.15 μg of DNA); Lane 8 (0.20 μg of DNA); Lane 9 (0.16 μg of DNA). (C) Twenty-hour incubation. Lanes 1–4 contain standards (as in A). HREC (0.18 μg of DNA/lane) in 25 m*M* glucose (Lanes 5 and 6), and in 5 m*M* glucose (Lanes 7 and 8). (D) HUVEC in 5 m*M* glucose: Lane 1 (0.11 μg of DNA); Lane 2 (0.13 μg of DNA); Lane 3 (0.22 μg of DNA); Lane 4 (0.20 μg of DNA). HUVEC in 25 m*M* glucose: Lane 5 (0.15 μg of DNA); Lane 6 (0.14 μg of DNA); Lane 7 (0.13 μg of DNA); Lane 8 (0.26 μg of DNA); Lane 9 (0.20 μg of DNA); Lane 10 (0.25 μg of DNA).

medium from pericytes incubated in 5 m*M* glucose (data not shown), either by the zymographic method described or in unfractionated medium tested by a fibrin plate method (kindly performed by Dr. P. Glas-Greenwalt). There were large differences between HUVEC and HREC in the quantity of 54-kDa PA in culture medium after 2 hr of incubation and in the effect of 25 m*M* glucose on PA (Fig. 5): Under basal conditions, HUVEC secreted significantly more PA than HREC (1.7-fold, $P < 0.05$). In contrast, 25 m*M* glucose stimulated 54-kDa PA secretion by HREC (60 $\pm$ 1%, $P < 0.05$), but not by HUVEC (5 $\pm$ 14%, NS).

Discussion

The loss of cellular constituents of the retinal capillaries is one of the earliest pathologic findings in background diabetic retinopathy. Hyperglycemia is one of the metabolic abnormalities thought to be responsible for development of acellular vessels with subsequent capillary closure (31–33). The present data demonstrate that an elevated glucose concentration comparable to that found in patients with diabetes diminishes proliferation of human retinal pericytes. Proliferation of human endothelial cells derived from both retinal microvessels and the umbilical vein was likewise reduced by this high glucose concentration. The extent of suppression was comparable in the latter two cell types. In contrast, proliferation of HRPE cells was not affected by the higher glucose concentration under these experimental conditions, demonstrating some specificity to this effect. The effect of glucose on the proliferation of HUVEC has been studied extensively by Lorenzi and co-workers (31, 32). The present findings in HUVEC are in agreement with those studies and do not show evidence that would limit the extrapolation of those findings to proliferation of HREC. While HREC and HUVEC manifest a similar proliferative response to glucose, they differ in their responses to growth hormone, which stimulates proliferation of HREC but does not alter proliferation of HUVEC (34).

Consistent with findings in pericytes of bovine origin, HRPC prove to be a 6-fold greater source of prostacyclin than endothelial cells, based on measurements of the metabolite 6-keto-$PGF_{1\alpha}$, whereas the endothelial cells prove to be the greater source of PGE_2 (35). The 6-keto-$PGF_{1\alpha}$ content in medium of HRPC in 25 m*M* glucose was 54% higher than that observed in 5 m*M* glucose. The PGE_2 content in medium from HREC grown in 25 m*M* glucose was 40% higher than that in the lower glucose control. In contrast, those cells secreting lesser amounts of either prostaglandin failed to respond to glucose. On the basis of these findings, HUVEC differ from HREC not only in the ability to produce PGE_2 under basal conditions, but also in the modulation of its secretion by glucose. In contrast to a previous report that a high glucose concentration de-

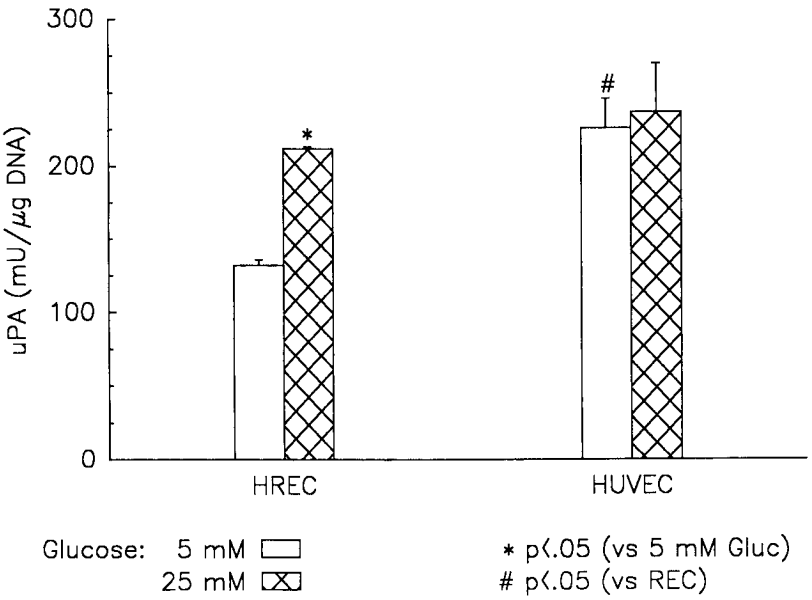

Figure 5. Effect of glucose on urokinase-type plasminogen activator in culture medium (2-hr incubation) of HREC (n = 4) and HUVEC (n = 5). Cells were plated as described in the legend to Figure 3.

creases PGI_2 production by cultured bovine aortic endothelial cells (11), in these experiments, 25 mM glucose did not significantly alter PGI_2 secretion by either type of endothelial cell. This apparent discrepancy likely reflects the regional specificities between these two vascular beds and may have implications for the differences in vascular pathology evoked by diabetes.

These opposite effects of glucose on pericyte secretion of prostacyclin and pericyte proliferation may have complex, but important, implications during the natural history of diabetes. Stimulation of HRPC secretion of prostacyclin on a per cell basis after a 9-day exposure to 25 mM glucose suggests that hyperglycemia may increase local prostacyclin concentrations in the retinal microcirculation in diabetes. In contrast, the fact that there is a loss of pericytes (the primary source of prostacyclin) in diabetes would decrease local prostacyclin concentrations. Early in the natural history of diabetic retinopathy, the glucose effect on prostacyclin secretion per cell is likely to be the predominant effect. However, as pericyte loss in local retinal vascular beds progresses, the net result of effects on pericyte number and function may be either normal, increased, or reduced local concentrations of prostacyclin. Finally, as pericyte loss becomes extreme, a net reduction in pericyte-derived prostacyclin in affected regions of the retinal microcirculation would be expected. This, in turn, would contribute to the abnormal flow and permeability characteristic of early diabetic microangiopathy, both by changing vascular tone and by accelerating formation of microvascular thrombi, and lead to capillary closure.

Substantial release of PGE_2 by HREC under basal conditions, and its augmentation by 25 mM glucose, may also have implications for the postulated role of this prostaglandin in vascular pathology. That PGE_2 stimulates platelet aggregation and increases vascular permeability is evidence that vascular cells are targets of this mediator (13, 15, 17, 18). These suggest that increased PGE_2 production by the retinal microvascular cells during hyperglycemia would be expected to favor capillary closure. In addition, PGE_2 has also been shown to stimulate angiogenesis (36), which may contribute to the proliferative stage of diabetic retinopathy.

The secretion of plasminogen activators is important in the activation of the fibrinolytic cascade. Plasmin generated by the fibrinolytic system activates latent collagenases necessary for basement membrane degradation. Such degradation is an initiating event in endothelial cell migration and proliferation leading to neovascularization (21–23). In the present study, PA activity was detected in the medium bathing both types of endothelial cells, maintained at the same cell density during a brief (2 hr) incubation under serum-free conditions. In contrast, no PA activity could be detected in cultures of HRPC. Under the conditions used here, both types of endothelial cell secreted plasminogen activators of molecular mass corresponding to the urokinase-type (54 kDa and 33 kDa). Occasionally, a faint band of lysis was evident at approximately 120 kDa, corresponding to a t-PA/PA inhibitor complex (5, 21); however, this was below the level necessary for quantitation. In contrast, predominant secretion of t-PA has been observed in other studies (5, 24, 37). There are several possible explanations for this discordance. Those we consider most plausible include: the cell

density at which the studies were conducted (subconfluent in these experiments versus confluent in the other reports); the composition of the culture medium (e.g., omission of heparin from the present studies because heparin alters the pattern of prostaglandins secreted); and the duration of incubation (2 hr in the present studies versus 5–48 hr in the others). Further studies will be necessary to distinguish these possibilities. Nevertheless, retinal endothelial cells secreted significantly less u-PA than did endothelial cells from the umbilical vein. Glucose (25 m*M*) augmented u-PA secretion from HREC, but not from HUVEC. This suggests that the vascular bed of origin may determine the expression of PA, and regulate PA activity by such metabolic perturbations as hyperglycemia. These regional differences are likely to impact on local PA activity, providing further evidence that measures of circulating PA activity in peripheral blood and their response to perturbations (38–40) may not predict the activity in any specific regional circulation such as the retinal microvasculature. The stimulation by glucose of HREC secretion of 54-kDa PA is consistent with an effect that would increase basement membrane proteolysis during periods of hyperglycemia.

In summary, microvascular pericytes from the human retina secrete large quantities of prostacyclin, whereas endothelial cells derived from the same site secrete large quantities of PGE_2 and urokinase-type PA under the conditions of study. These mediators, which are candidates for roles in the early stages of human diabetic retinopathy, are all modulated by glucose. Differences between retinal microvascular endothelial cells and HUVEC with respect to the level of secretion and control by glucose of these species provide evidence of regional specificity of these functions. These may have consequences for differences in manifestations of diabetic vascular disease in large and small vessels and provide a basis for caution in the selection of human endothelial cells for *in vitro* studies concerning diabetic retinal microvascular disease.

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