Elevated Glucose Concentrations Increase Factor VIIIR:Ag Levels in Human Umbilical Vein Endothelial Cells

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SUMMARY

To determine if endothelial cell metabolism is affected by the elevated glucose concentrations known to occur in diabetes mellitus, we measured cellular Factor VIIIR:Ag in endothelial cells grown under conditions of increased glucose concentration. The results of this study illustrate that, under cell culture conditions, an increase in glucose concentration results in increased cellular levels of Factor VIIIR:Ag. Specifically, a glucose concentration of 300 mg/dl resulted in a 23% increase in Factor VIIIR:Ag levels while a glucose concentration of 600 mg/dl resulted in a 54% increase in Factor VIIIR:Ag levels. These in vitro findings may relate to the increase in plasma Factor VIIIR:Ag levels known to occur in diabetic patients. DIABETES *32*:876–878, September 1983.

It has been well-documented that the patient with diabetes mellitus has an increased frequency and severity of vascular disease. These findings have led many research groups to initiate in-depth studies of the coagulation system in an attempt to identify those portions involved in the development of occlusive vascular disease in diabetes mellitus. Factor VIII coagulant (VIII:C), Factor VIII-related antigen (VIIIR:Ag), and the von Willebrand/Ristocetin co-factor (VIII:Rcof)[1-4] have been reported to be elevated in patients with diabetes mellitus, while the fibrinolytic activity was depressed or normal.[5] In all studies to date it has been difficult to determine if these changes lead to or are caused by the vascular disease.

Since Factor VIIIR:Ag is synthesized in endothelial cells, the plasma level serves as a useful marker for intimal damage.[6] The rationale for increased levels of Factor VIIIR:Ag rests on the hypothesis that the endothelium (the cellular source of this protein) when injured in diabetic angiopathy synthesizes and/or releases increased amounts of this protein.[3,4] In diabetics, increased platelet sensitivity has been shown to correlate with elevated levels of Factor VIIIR:Ag.[7,8] Increased Factor VIIIR:Ag may be one of the factors together with abnormal lipid metabolism that leads to increased platelet adhesion, aggregation, and eventual thrombosis formation. Alternatively, the increase in the plasma level of Factor VIIIR:Ag could be due to a simple metabolic aberration of the endothelium caused by the diabetic state and not be initially due to a physical injury of the endothelium. To date there has been little evidence to suggest that endothelial synthesis and/or release of Factor VIIIR:Ag is related to hyperglycemia, the hallmark of diabetes mellitus.

To determine if endothelial metabolism of Factor VIIIR:Ag is affected by the diabetic milieu we placed endothelial cells into culture where the response to alterations in glucose concentration could be tested. The results of this determination clearly demonstrate that an increase in glucose concentration can alter the cellular level of Factor VIIIR:Ag present in endothelial cells.

MATERIAL AND METHODS

Isolation and culture of human umbilical vein endothelial cells. Human umbilical cords were obtained immediately after normal vaginal delivery. The umbilical vein in undamaged segments of cord (20–30 cm in length) was identified, cannulated, and rinsed 3× with phosphate-buffered saline (PBS) to remove residual blood. The vein was then filled with 1 mg/ml collagenase (Sigma Chemical Co., St. Louis, Missouri, lot 52F6819) in PBS and incubated at 37°C for 20 min. The liberated endothelial cells were concentrated by centrifugation and resuspended into Dulbecco's Modified Eagles' Medium (DME) containing 150 μg/ml of endothelial mitogen (Biomedical Technologies, Cambridge, Massachusetts), 10 μg/ml transferrin (Collaborative Research, Lexington, Massachusetts), and 10% vol/vol human serum. The

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TABLE 1
Variation of intracellular levels of Factor VIIIR:Ag as a function of umbilical cord isolate

Umbilical cord isolate	Flask 1	Flask 2	Flask 3	Mean ± SEM
1	1.92	1.71	1.41	1.68 ± 0.15
2	3.10	3.80	3.15	3.35 ± 0.23
3	1.50	1.27	1.50	1.42 ± 0.08
4	0.30	0.48	0.81	0.53 ± 0.15
5	2.10	2.90	2.60	2.53 ± 0.23
6	2.88	2.58	2.85	2.77 ± 0.10
7	1.05	0.91	1.45	1.14 ± 0.16
8	2.75	3.35	3.30	3.13 ± 0.20
Total (mean ± SEM)	1.95 ± 0.34	2.13 ± 0.42	2.13 ± 0.34	2.07 ± 0.36

The procedures for the isolation, culture, and determination of cellular levels of Factor VIIIR:Ag of endothelial cells were as described in MATERIALS AND METHODS. The cellular levels of Factor VIIIR:Ag from each cord was determined in triplicate from each of a series of triplicate 25-cm² T-flasks. The variation in triplicates from each individual flask was less than 13%. Factor VIIIR:Ag is expressed as U/dl.

cells were plated into a 25-cm² T-flask with the growth surface containing a bovine type 1 collagen coat (Collaborative Research). Under these conditions the endothelial cells proliferate to confluence within 48–96 h and can be subcultured at a 1:3 ratio for 13 additional passages before cell senescence occurs.

In order to more closely reflect the metabolic conditions seen in humans, endothelial cells were grown in medium containing human serum. Human serum was prepared by collecting whole blood in glass tubes from normal volunteers. The blood was allowed to clot and retract at 4°C for 24 h. The serum was decanted and centrifuged at 1800 × *g* to remove residual RBCs. The serum was heat inactivated at 56°C for 30 min. The sera from 10 normal volunteers were tested for the ability to support endothelial cell growth. The most active serum was used for the studies reported herein and in no case was pooled serum used.

The effect of varying glucose concentration on Factor VIIIR:Ag production was assessed employing endothelial cells at passage 3. For each experiment a confluent culture at passage 2 (a 75-cm² T-flask) was harvested using trypsin-EDTA (0.05%:0.02%); the reaction was then halted by the addition of an equal volume of human serum and the cells collected by centrifugation. The cells were then equally distributed among nine 25-cm² T-flasks at a 1:3 subculture ratio. Triplicate flasks contained medium with glucose concentrations of 100, 300, and 600 mg/dl. The cells were fed fresh growth medium every 3 days. The number of cells per cm² at confluence did not vary as a function of glucose concentration.

To determine the response of the cellular level of Factor VIIIR:Ag to glucose concentration the cells were grown to confluence in the above media. On day 7 (1 day after the change of growth medium) the growth medium was removed and the monolayer washed 5× with 5-ml aliquots of PBS. One milliliter of PBS was then added to the flask and the cells removed using a rubber policeman. The cells were transferred to a Potter Elvehjem homogenizer and disrupted by 30 strokes of the pestle. All procedures were performed at 4°C and the flask checked for complete monolayer removal by light microscopy. Cell disruption was checked by trypan-blue staining with examination by microscopy and was found to be greater than 95%. The cell homogenates were stored frozen at −70°C until assayed.

Assay of Factor VIIIR:Ag. The cellular level of Factor VIIIR:Ag was determined as described previously, employing a rabbit antibody monospecific for human Factor VIIIR:Ag.[9] One unit of Factor VIIIR:Ag is the amount present in 1 ml normal plasma. The assay is sensitive to Factor VIIIR:Ag levels as low as 0.3 U/dl.

RESULTS

To establish the basal levels of Factor VIIIR:Ag in human umbilical vein endothelial cell cultures eight umbilical cords were assessed. The results of this determination (Table 1) clearly demonstrated that the cellular levels of Factor

TABLE 2
Effect of elevated glucose concentration on cellular levels of Factor VIIIR:Ag in endothelial cells

Umbilical cord isolate	Factor VIIIR:Ag Glucose (100 mg/dl)	Glucose (300 mg/dl)	Glucose (600 mg/dl)	Percent change 100 mg/dl vs. 300 mg/dl	100 mg/dl vs. 600 mg/dl
1	1.68	2.10	2.20	25.0	31.0
2	3.35	4.20	4.12	25.4	23.0
3	1.42	1.78	2.20	25.4	54.9
4	0.53	0.61	1.00	15.0	88.7
5	2.53	3.15	4.47	24.5	76.7
Mean ± SEM	1.90 ± 0.48	2.37 ± 0.61	2.80 ± 0.65	23.1 ± 2.0	54.9 ± 12.6

The cellular level of Factor VIIIR:Ag in endothelial cells as a function of glucose concentration in the growth medium was assessed as described in MATERIALS AND METHODS. Factor VIIIR:Ag is expressed as U/dl. Using Student's paired *t* test, there was a significant difference between cellular Factor VIIIR:Ag in cells grown in glucose at 300 mg/dl or 600 mg/dl when compared with the control ($P < 0.05$).

VIIIR:Ag vary as a function of the umbilical cord from which they were isolated. That this variation was due to the umbilical cord obtained and not culture conditions was strongly suggested by the finding that triplicate cultures from the same cord demonstrated stable cellular levels of Factor VIIIR:Ag (Table 1). The mean cellular levels of Factor VIIIR:Ag among individual umbilical cord isolates varied from a low value of 0.53 to a maximum value of 3.35, with a value of 2.07 representing the mean of all determinations.

The endothelial cell cultures from five of the above isolates were also assessed for the effect of elevated glucose concentrations in the growth medium with regard to cellular Factor VIIIR:Ag levels. Experiments were performed to insure that changes in glucose concentration did not affect the ability to measure Factor VIIIR:Ag. The elevation of glucose from 100 mg/dl to 300 mg/dl resulted in an increase of cellular Factor VIIIR:Ag levels by approximately 23% (Table 2). A further increase in glucose concentration to 600 mg/dl resulted in a rise in Factor VIIIR:Ag levels by 54% compared with the 100 mg/dl control (Table 2). Factor VIIIR:Ag levels were also measured in the conditioned culture medium to assess whether Factor VIIIR:Ag released by endothelial cells could be monitored. The levels were 14.30 ± 1.79 at 100 mg/dl, 14.14 ± 2.25 at 300 mg/dl, and 13.76 ± 2.36 at 600 mg/dl. There were no statistically significant changes found at the various glucose concentrations. The presence of extraneous Factor VIIIR:Ag (in 10% human sera) in the growth medium limited our ability to detect small changes of released Factor VIIIR:Ag that may have occurred.

DISCUSSION

The results of the present study clearly illustrate that under cell culture conditions, an increase in glucose concentration (to 300 or 600 mg/dl) results in an increased level of Factor VIIIR:Ag in endothelial cells. To our knowledge this represents the first study that demonstrates this phenomenon. The significance of this finding is strengthened by the in vivo observation that the increased plasma levels of Factor VIIIR:Ag occur in patients with diabetes mellitus before clinically apparent manifestations of vascular disease develop (i.e., retinopathy).[1-4] This suggests that in vivo the elevation of Factor VIIIR:Ag levels may be due, at least in part, to the hyperglycemia of diabetes mellitus.

It is recognized that only modest elevations of Factor VIIIR:Ag levels (mean 23%) were seen with glucose concentrations in the range usually encountered in uncontrolled diabetes mellitus. However, it is possible that prolonged exposure of the endothelium to this concentration of glucose in vivo could lead to a chronic increase in Factor VIIIR:Ag levels. The demonstration that elevated glucose levels lead to an increased level of Factor VIIIR:Ag in endothelial cells has several possible explanations. The Factor VIIIR:Ag measured could have undergone proteolytic degradation, which would result in elevated values in the antibody-based assay system employed. This possibility was eliminated by the demonstration that the Factor VIIIR:Ag was of the expected size distribution and was not degraded (manuscript in preparation). Other possibilities include uptake of Factor VIIIR:Ag from the growth medium, increased storage, or increased synthesis. These possibilities cannot be separated by the limited studies performed by us or from studies performed in diabetic patients by other investigators. While the specific uptake of Factor VIIIR:Ag has been reported by one research group,[10] a more recent study by another research team failed to demonstrate the presence of Factor VIIIR:Ag receptors on these cells.[11] There is limited evidence for increased storage or synthesis of Factor VIIIR:Ag by endothelial cells in vivo. The studies of Giustolisi and co-workers postulate that the increase in plasma Factor VIIIR:Ag levels in diabetic patients after several hours of venostasis occurs through the mechanism of increased synthesis and release of stored Factor VIIIR:Ag.[12] Our preliminary studies showing an increase in cellular Factor VIIIR:Ag levels with increasing glucose concentration are in agreement with either increased storage and/or synthesis. To elucidate which of the above mechanisms are responsible for the increased levels of Factor VIIIR:Ag found in our preliminary studies will require isotopic labeling and pulse-chase experiments. These studies are presently being undertaken.

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