D-Glucose and insulin stimulate migration and tubular formation of human endothelial cells in vitro

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Shigematsu, Satoshi, Keishi Yamauchi, Kohji Nakajima, Sachiko IIjima, Toru Aizawa, and Kiyoshi Hashizume. D-Glucose and insulin stimulate migration and tubular formation of human endothelial cells in vitro. Am. J. Physiol. 277 (Endocrinol. Metab. 40): E433-E438, 1999.—Effects of high D-glucose and insulin on the endothelial cell migration and tubular formation were investigated with the use of ECV304 cells, a clonal human umbilical cord endothelial cell line. Exposure of the cells to high D-glucose resulted in a marked increase in the migration, which was blocked by inhibitors of protein kinase C such as H7 (10 µM) and GF109203X (200 nM). Furthermore, a protein kinase C agonist, phorbol 12-myristate 13-acetate, had an effect similar to that of glucose on ECV304 cells. Glucose stimulation of the migration was additively enhanced by 100 nM insulin, and the insulin effect was found to be unaffected by either PD-98059 or wortmannin, a mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase inhibitor and a phosphatidylinositol 3-kinase inhibitor, respectively. Neither did H7 inhibit insulin stimulation of the migration. In contrast, a combination of high D-glucose and insulin, rather than either one alone, promoted tubular formation, which was inhibited by addition of 10 µM PD-98059. Stimulation of ECV304 cells by the combination of high D-glucose and insulin also caused an activation of MAPK, which was again obliterated by the same concentration of PD-98059. In conclusion, human endothelial cell migration and tubular formation are stimulated by high D-glucose and insulin in different ways. In the former reaction, either is effective, a combination of the two results in an additive effect, and activation of protein kinase C is involved. In contrast, tubular formation will only occur in the presence of a combination of high D-glucose and insulin, and MAPK plays an essential role.

The mechanism of the development and progression of diabetic vascular complication is only poorly understood. Clinical observation indicates that both hyperglycemia and, to a lesser degree, hyperinsulinemia are risk factors (1, 11, 22, 25, 26, 29). Although the exact cellular and subcellular mechanisms of hyperglycemia- and hyperinsulinemia-induced vascular injury have not as yet been very clearly delineated, there is at least a consensus that the process is quite complex and mediated by multiple intracellular signals (18). The involvement of various growth factors is also indicated in previous in vitro studies (3, 8, 16, 20). As far as vascular biology is concerned, one of the principal components of diabetic vascular complication is an angiogenesis in which the migration and tubular formation of the endothelial cells are the pivotal events (21, 23, 24). On the basis of these facts, we systematically examined the cellular and subcellular effects of D-glucose and insulin in relation to the migration and tubular formation of the endothelial cells to obtain an insight into how they are activated in the diabetic milieu.

**Materials and Methods**

Cell culture. ECV304 cells (a clonal human umbilical cord endothelial cell line) were obtained from Riken Cell Bank (Tokyo, Japan) and cultured in RPMI 1640 (GIBCO Laboratory) supplemented with 10% fetal bovine serum (GIBCO), 100 mg/ml crude endothelial cell growth factor (GIBCO), and 5 U/ml heparin (Novo Nordisk) at 37°C with 5% CO2.

Chemicals. Human recombinant insulin was obtained from Eli Lilly (Indianapolis, IN). D-Glucose, L-glucose, phorbol 12-myristate 13-acetate (PMA), and wortmannin were purchased from Calbiochem (La Jolla, CA). Anti-mitogen-activated protein kinase (MAPK) polyclonal antibody (α-MAPK), anti-phospho-specific MAPK polyclonal antibody (α-pMAPK), anti-AKT polyclonal antibody (α-AKT), and anti-phospho-specific Akt polyclonal antibody (α-pAkt) were obtained from New England Biolabs (Beverly, MA). FK-366 is a generous gift from Fujisawa Pharmaceutical (Osaka, Japan).

Cell migration assay. Cell migration assay was performed as previously described (19) with minor modifications. Migration was determined in 24-well plates with 8.0-µm-pore-size cell culture inserts. Subconfluent ECV304 cells were harvested with trypsin-EDTA, washed, and resuspended at 1 x 10^6 cells/ml in Ham's F-12K solution (GIBCO) with 1% fetal bovine serum, and a 0.2-ml aliquot was placed onto each insert. The inserts were then placed in the wells containing 0.7 ml serum-free Ham's F-12K with type 1 collagen (Sigma; 3 µg/ml). The cells were incubated for 4 h, allowing migration through the 8-µm pores of the inserts, and the effects of various concentrations of sugars, insulin, and pharmacological agents were determined. The following pharmacological agents were used at the concentrations indicated: H7 (10 µM) and GF109203X (200 nM), protein kinase C (PKC) inhibitors; PMA (100 nM), a PKC agonist; wortmannin, a phosphatidylinositol (P1) 3-kinase inhibitor; and PD-98059 (10 µM), a MAPK/extracellular signal-regulated kinase (MEK) inhibitor. The migration was quantified as follows. All nonmigrated cells were removed from the upper surface of the insert membrane with a scraper, and the migrated cells, attached to the lower surface, were fixed and stained with May-Grunwald and Giemsa solutions. The numbers of stained cells were counted with a microscope at a magnification of X200.
Tubular formation assay. Confluent ECV304 cells were washed with PBS and cultured in serum-free Ham's F-12K solution with test substances and pharmacological agents for 3 days. Then the cells were stained with May-Grunwald and Giemsa solutions to visualize the tubular formation.

Western blot analysis. Whole cell extracts were prepared by exposing the cells to lysis buffer (20 mM HEPES, pH 7.4, 1% Triton X-100, 2 mM EDTA, 2 mM EGTA, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 µM leupeptin, 10 µg/ml aprotinin, and 1.5 µM pepstatin) for 1 h at 4°C. The resultant cell extracts were electrophoresed as described (31) and subjected to Western blotting with the use of antibodies such as α-MAPK, α-pMAPK, α-Akt, and α-pAkt; they were visualized with the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

Statistical analysis. Statistical significance of difference was evaluated by using ANOVA with Fisher's protected least significant difference test (StatView; SAS Institute, Cary, NC). P < 0.05 was considered statistically significant.

RESULTS

Effects of high D-glucose and insulin on the migration of ECV304 cells. We first examined the effects of various sugars on the migration. As shown in Fig. 1, high concentrations of both levels of D-glucose strongly stimulated the migration of ECV304 cells, whereas L-glucose, a nonmetabolizable sugar, only minimally stimulated the migration at a very high concentration. Therefore, we concluded that it was not medium hyperosmolarity but D-glucose-derived metabolic signals generated in the cell that were mostly, if not entirely, responsible for the exaggerated migration.

PKC is implicated as a mediator of high D-glucose-induced cellular activation in diabetes (7, 15), and this is presumed to be due to increased de novo production of diacylglycerol as a consequence of excessive D-glucose metabolism (18). Accordingly, we examined the effects of PKC inhibitors such as H7 and GF109203X and a PKC agonist, PMA, on the migration. H7 (10 µM) and GF109203X (200 nM) clearly suppressed D-glucose-stimulated migration (Fig. 2). PMA potently enhanced cell migration in the medium containing 7 mM D-glucose (Fig. 2). On the other hand, high D-glucose (23.7 mM) did not further enhance PMA-stimulated migration. Thus PKC is probably a mediator in the D-glucose stimulation of the migration of ECV304 cells.

In the next stage, we examined the effects of insulin. As shown in Figs. 3 and 4, insulin stimulated the migration in both low- and high-glucose media. The stimulatory effect of insulin on the migration was not inhibited by wortmannin, a PI 3-kinase inhibitor, or by PD-98059, an inhibitor of MEK. Although H7 inhibited basal and insulin-induced migration of ECV304 cells, insulin still effectively stimulated the migration.

Effects of high D-glucose and insulin on the tubular formation of ECV304 cells. As shown in Fig. 5, a high concentration of D-glucose in combination with 100 nM insulin strongly promoted tubular formation of the
cells. On their own, however, neither high $\alpha$-glucose nor insulin had any significant effect, nor did nonmetabolizable sugars such as $\beta$-glucose or sucrose, either alone or in combination with insulin. Therefore, it is not medium hyperosmolarity but $\alpha$-glucose-derived metabolic signals generated in the cell and insulin that stimulated tubular formation of ECV304 cells.

MAPK, a downstream signaling molecule of insulin, is mostly, if not entirely, responsible for mediating growth promotion of insulin (6), whereas Akt, another insulin-signaling molecule, downstream from PI 3-kinase, activates glycogen synthesis by inactivating glycogen synthase kinase-3, so that it is a key molecule mediating the metabolic effect of insulin (5). An inhibitor of MEK, PD-98059 (10 $\mu$M), suppressed tubular formation of ECV304 cells induced by a combination of high $\alpha$-glucose and insulin (Fig. 6). The same concentration of PD-98059 clearly blocked MAPK activation induced by the combination of high glucose and insulin (Fig. 6). The same concentration of PD-98059 clearly blocked MAPK activation induced by the combination of high glucose and insulin, but it did not alter the insulin-induced activation of Akt (Fig. 7). H7 and wortmannin had no effect on the tubular formation promoted by insulin and high $\alpha$-glucose (data not shown).

**DISCUSSION**

Endothelial cell migration and tubular formation constitute, respectively, the initial and secondary stages in angiogenesis (27). Accordingly, the effects of high $\alpha$-glucose and insulin on these two processes were systematically analyzed in the present study with the use of human endothelial cells in vitro. The following important facts were found.

First, we were able to demonstrate conclusively for the first time that high $\alpha$-glucose and insulin are responsible for direct stimulation of the migration and tubular formation. In addition, the mode of action of high glucose and insulin was shown to be distinctively different in the two processes. Namely, high glucose and insulin stimulated the former process independently, and a combination of the two had an additive effect on it. In contrast, a combination of high glucose and insulin was needed to promote tubular formation.

Second, with regard to mediation of the glucose stimulation of the migration, activation of PKC appeared to be important because both H7 and GF109203X inhibited the migration. Although H7 inhibits both...
PKC and protein kinase A at a concentration of 10 µM, 200 nM GF109203X does not inhibit protein kinases other than PKC (13, 14). Furthermore, PMA, a PKC agonist, clearly stimulates migration of ECV304 cells in the presence of low glucose. As the maximum glucose did not show a synergistic effect on top of PMA effect, it may partially activate PKC. Activation of PKC has been implicated in the development of diabetic complications in many studies (7, 15), and the enzyme has been shown to affect diverse vascular functions (4). The mediation of the insulin stimulation of migration was not clarified because none of the inhibitors tested in the present study suppressed this insulin action. However, the failure of wortmannin to suppress insulin stimulation of the migration strongly indicates that PI 3-kinase does not play an important role.

Third, a combination of glucose and insulin appeared to promote tubular formation of ECV304 cells by means of activation of MAPK, an insulin-receptor downsignaling molecule located distal to the Shc/Grb2 branch. On one hand, activation of MAPK elicited by a combination of high glucose and insulin in the cells was effectively blocked by PD-98059, a MEK inhibitor, whereas, at the same concentration, the drug obliterated promotion of tubular formation of the cells caused by high glucose and insulin. However, insulin alone failed to promote the tubular formation. This indicates that activation of MAPK is required but not sufficient by itself to promote tubular formation of ECV304 cells. Only when insulin stimulation and high-glucose stimulation are combined is tubular formation of the cells promoted. In relation to this, a cross talk between the PKC branch and insulin-receptor downsignaling is suggested by Haneda et al. (10), who demonstrated that hyperglycemia stimulates the MAPK pathway through PKC activation in mesangial cells. However, our finding that H7 failed to inhibit the tubular formation elicited by a combination of high glucose and insulin suggests that involvement of PKC is unlikely in the tubular formation of ECV304 cells.

Our data do not directly rule out the possibility of other high glucose-induced processes, including polyol accumulation (17), nonenzymatic glycation of proteins (9, 12, 28), and an alteration of redox potential (30), as mediators of the increased migration of the endothelial cells. However, we think polyol accumulation is most unlikely as a mediator of the increased migration for the following reasons. The increase in migration was demonstrable as early as 2 h post-exposure to high glucose, and the treatment of the cell with FK-366, a potent aldose reductase inhibitor (2), had no suppressive effect whatsoever (data not shown).

Although we do not know whether the endothelial cell migration and tubular formation seen in the in vitro experiments are faithfully mimicking the early stages of diabetic vascular complications in vivo, the activation of ECV304 cells observed in the present study can at least serve as an experimental model to study the nature of angiogenesis in diabetes.

Our finding that glucose or insulin alone stimulates the endothelial cell migration, the initial stage of angiogenesis, and that a combination of the two is required for tubular formation, the ensuing process, is intriguing. First, the finding is in accord with the clinical evidence that hyperglycemia itself is a strong promoter of diabetic microangiopathy (1, 11, 22, 26). On the other hand, there is no direct evidence to suggest that hyperinsulinemia is an independent risk factor for
the development and progression of microangiopathy. In obese, nondiabetic subjects, plasma insulin is elevated more than in patients with type 2 diabetes because of insulin resistance, but plasma glucose is not. Macroangiopathic complication is less prevalent in such subjects than in patients with diabetes, indicating that patients with a combination of hyperglycemia and hyperinsulinemia, but not either one alone, are especially prone to develop macroangiopathy. Diabetic microangiopathy is definitely more common in insulin-treated than in non-insulin-treated patients with diabetes (25, 29), suggesting that, here again, a combination of hyperinsulinemia and hyperglycemia promotes development of the angiopathy. Because the data were obtained with the use of umbilical endothelial cells, our findings may have relevance to fetal and placental abnormalities in diabetic pregnancy as well.

In conclusion, high-o-glucose and insulin stimulate both migration and tubular formation of the endothelial cells, and involvement of PKC and MAPK, for the former and the latter processes, respectively, was strongly implicated. These findings provide a cellular basis for explaining exaggerated angiogenesis in the diabetic milieu.

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