

DYS-REGULATION OF EXTRACELLULAR MATRIX PROTEINS TURNOVER BY HIGH GLUCOSE CONCENTRATIONS IN CULTURED HUMAN GLOMERULAR MESANGIAL CELLS

ROHOLLAH HOSSEINI^{*}, GERHARD HAMPEL^{**} and KLAUS JUNG^{**}

^{*}Department of Toxicology, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, IRAN.

^{**}Research Division of Urology Department, Faculty of Medicine, Humboldt University of Berlin, Berlin, GERMANY.

ABSTRACT

Diabetic glomerulopathy is generally believed to be the major cause for the development of chronic renal failure in diabetes mellitus. Diabetic glomerulosclerosis is characterized by the accumulation of extracellular matrix proteins such as fibronectin and type IV collagen in the mesangium. In this study, the effect of high glucose (33.6 mM) on fibronectin and type IV collagen concentrations in the supernatant of human glomerular mesangial cell culture was studied. The concentrations of fibronectin and type IV collagen in tissue culture supernatant, assayed by ELISA techniques increased significantly ($P < 0.01$) by the high glucose level (33.6 mM) after 6 days incubation. Direct cell counting and thymidine incorporation methods showed that high glucose concentrations (33.6 mM, 56 mM and 112 mM) inhibit the mesangial cell (MC) proliferation in concentration-dependent manner. To study the osmotic effect of high glucose concentrations, the mesangial cells were also cultured in the presence of mannitol and it was found that mannitol did not have effect on cellular proliferation but increased fibronectin and type IV collagen concentrations significantly ($P < 0.05$) in the supernatants. These results indicate that the increase of synthesis and/or decrease degradation of fibronectin and type IV collagen by MCs may, in part, result from changes in osmolarity induced by high glucose concentration. These results suggest that elevation of fibronectin and type IV collagen production and/or decrease their degradation by the mesangial cells may play an important role in the accumulation of these extracellular matrix proteins which is common to diabetic glomerulosclerosis.

Key Words: Glomerulosclerosis, Mesangial Cells, High Glucose, Fibronectin, and Collagen.

INTRODUCTION

The glomerular mesangial cell (MC) plays a central role in the pathogenesis of diabetic nephropathy (1) and abnormalities in its growth and production of the extracellular matrix (ECM) correlate with the progressive decline in glomerular filtration rate (GFR) during the course of diabetic renal disease (2). The mesangial matrix is normally composed of various glycoproteins including fibronectin (FN) and type IV collagen (COL), which have been reported to increase in diabetic mesangium (3,4). It has also been reported that the MCs are responsible for the synthesis and metabolism of FN and COL in mesangial matrix (5). Increased

synthesis and/or decreased degradation of FN and COL by MCs could result in the accumulation of matrix, leading to glomerulosclerosis. Therefore, the ability of MCs to produce FN and COL under high glucose concentrations was investigated by measurement of these glycoproteins in the cell culture supernatants.

MATERIALS and METHODS

Reagents. Amphotericin B, Penicillin, streptomycin, DNA (from Calf Thymus), human fibronectin and type IV collagen were purchased from Sigma Chemical Co. (St. Louis, MO). Fetal Calf Serum (FCS) and PBS were from

RPMI 1640 with L-glutamine was from BioWhittaker (MD, USA). Hoechst 33258 were purchased from Fluka (Deisenhofen, Germany).

Isolation and culture of glomerular mesangial cells. Human mesangial cells were isolated from primary glomerular explants of the nephrectomized patients with kidney tumor by a graded-sieve technique. The cells were plated from culture in RPMI 1640 tissue culture medium supplemented with 20% (V/V) FCS, 100 IU/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B. For outgrowth, the mesangial cells were plated in 75-cm² flasks and incubated at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. In the present study, the mesangial cells with passage number between 6 to 10 were used.

Evaluation of glucose concentration on cellular proliferation. In order to assess the effect of glucose concentration on MCs proliferation, human MCs were exposed for up to 12 days to 5.6 mM, 33.6 mM and 112 mM glucose and 28 mM manitol + 5.6 mM glucose as control group for hypertonicity. Beginning at day 0, cellular proliferation was assessed every 48 hours by direct counting method using hemocytometer. In order to evaluate the cell growth using an alternative method, incorporation of tritiated thymidine by the human MCs was also examined. In this study, the MCs were incubated for up to 6 days in 96 well plates with the media containing 5.6 mM, 56 mM and 112 mM glucose and 50.4 mM manitol + 5.6 mM glucose as control group of hypertonicity.

Fibronectin (FN) and type IV collagen (COL) levels in tissue culture supernatants. To evaluate the effect of high glucose levels on FN and COL concentrations in the supernatants, the mesangial cells were cultured in 25 cm² flasks with media containing 5.6 mM and 33.6 mM glucose or 28 mM manitol + 5.6 mM glucose as control group of hypertonicity. All media were changed every 48 hours. On days 4, 6, 8, 10 and 12, FN and COL concentrations in the supernatants were measured using ELISA techniques, and then calculated on the basis of DNA content (ng and µg /µg of DNA, respectively).

Measurement of DNA. For assay of DNA, the harvested cells were lysed by ultrasonication and then DNA contents were measured by

spectrofluorometric method, using Hoechst 33258 dye, at the wavelengths of 355 nm for excitation and 460 nm for emission.

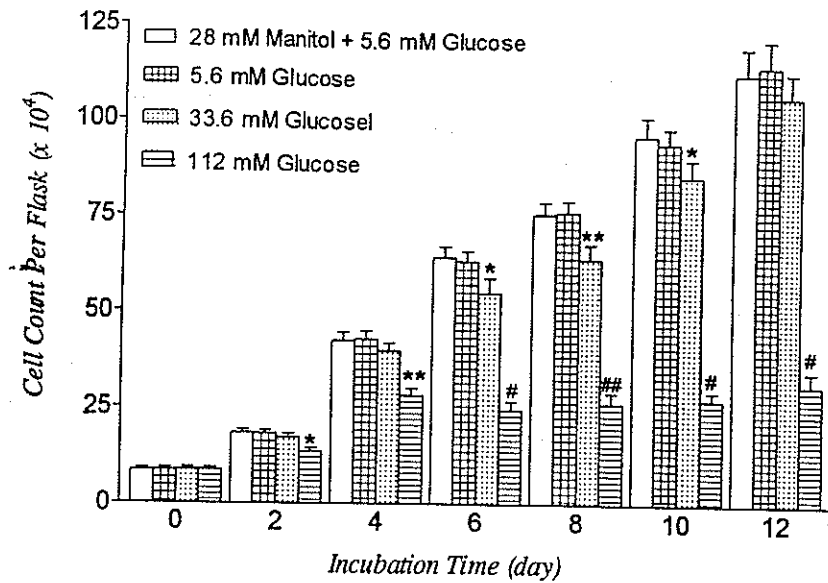
Statistical Methods. The results were presented as the mean ± SEM. The groups were compared by Student's *t*-test and Mann-Whitney *U*-test, using the GraphPad software version 2.01 (GraphPad Software Inc, San Diego, CA). Differences were considered significant at $P < 0.05$.

RESULTS

Effect of glucose on human mesangial cell proliferation. To study the effect of glucose on MC proliferation, the effect of different glucose concentrations (5.6 mM, 33.6 mM and 112 mM) on the cell growth were investigated. Our results showed that high glucose concentrations (33.6 mM and 112 mM) resulted in a significant decrease of cell numbers and this effect was more pronounced with the higher concentration of glucose (Figure 1). There was no significant effect of glucose or manitol on cell viability, since in all experiments, over 95% of the cells excluded by the exclusion of trypan blue. However, the results indicated that the effect of glucose on MC proliferation is concentration-dependent. Moreover, our results showed that exposure of the MCs to high glucose concentrations (56 mM and 112 mM) led to a significant decrease in the cell proliferation as compared to normal glucose (5.6 mM) based upon reduced incorporation of [³H]-thymidine, significantly (Figure 2). The inhibitory effect of elevated ambient glucose on MC proliferation was not a consequence of hypertonicity of the medium, since 50.4 mM manitol + 5.6 mM glucose did not reproduce the effect of high glucose concentration (56 mM).

Effect of glucose on tissue culture supernatant Fibronectin (FN) and type IV collagen (COL) concentrations. To assess effects of glucose on FN and COL production, the MCs were exposed up to 12 days to 5.6 mM and 33.6 mM glucose or 28 mM manitol + 5.6 mM glucose as control group of hypertonicity. The results are shown in figures 3 and 4 in which each data point represents total FN or COL productions by the MCs. Because total FN and COL concentrations in the supernatant will vary with cell number, the results were expressed on the basis of DNA content. As it can be found by the inspection of the provided figures, high glucose concentration

Figure 1. Effects of High Glucose Concentrations on the Mesangial Cells Growth as Evaluated by Direct Counting Method. * $P < 0.05$, ** $P < 0.01$, # $P < 0.001$ and ## $P < 0.0001$: As compared to normal (5.6 mM) glucose and manitol (28 mM manitol + 5.6 mM glucose) groups. Each bar represents the mean \pm SEM of 7 flasks. For more details, see Materials and Methods.



(33.6 mM) could significantly increase concentrations of FN and COL into the supernatants. To evaluate the effects of hypertonicity resulting from high glucose concentration on FN and COL levels, the effect of manitol (28 mM) + glucose (5.6 mM) on these glycoproteins levels in the cell culture supernatants was studied. As it is shown in figure 3, manitol as compared to normal glucose (5.6 mM) group could increase FN concentration after 6, 8, 10 and 12 days incubation, significantly ($P < 0.05$), but this effect is less than the effect of high glucose concentration

(33.6 mM). Manitol (28 mM) + glucose (5.6 mM) could also increase COL concentration significantly ($P < 0.05$) into the supernatants by MCs, only after 6 and 8 days. On the other hand, the effect of manitol on FN concentration was more pronounced and sustained than COL concentration.

DISCUSSION

Diabetic glomerulosclerosis is characterized by the accumulation of ECM proteins in the mesangium (6) and previous studies have demonstrated that the degree of mesangial

Figure 2. Thymidine Incorporation by Human Glomerular Mesangial Cells in Media Containing 5.6 mM, 56 mM and 112 mM Glucose or 50.4 mM Manitol + 5.6 mM Glucose. * $P < 0.05$, ** $P < 0.01$, # $P < 0.001$ and ## $P < 0.0001$: As compared to normal (5.6 mM) glucose and manitol (50.4 mM manitol + 5.6 mM glucose) groups. Data are presented as mean \pm SEM of 8 wells. For more details, see Materials and Methods.

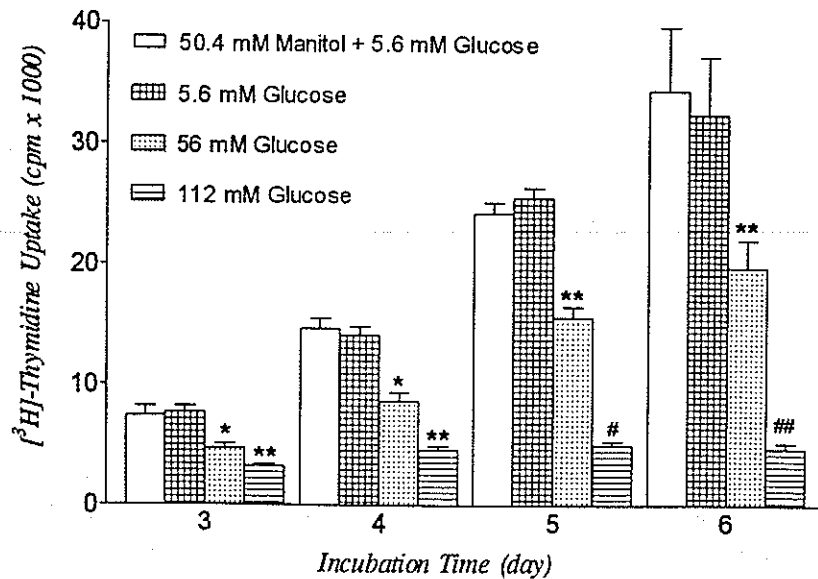
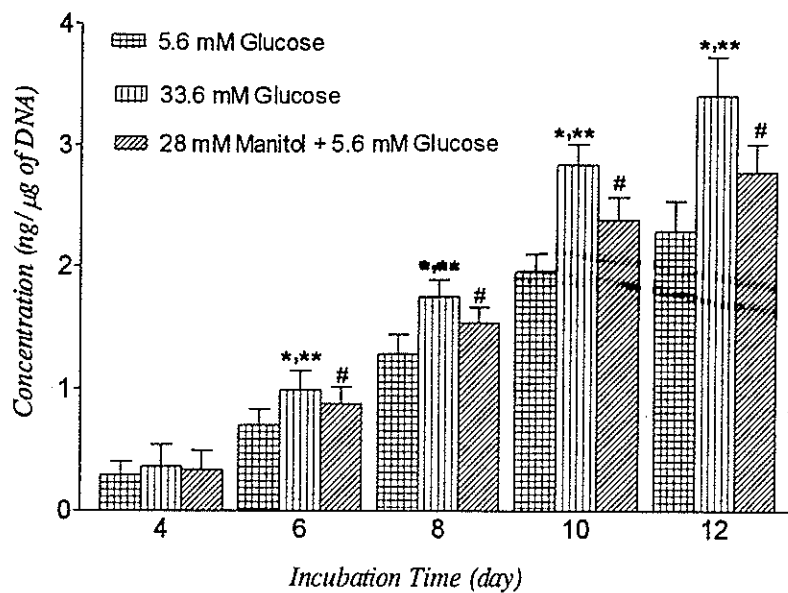


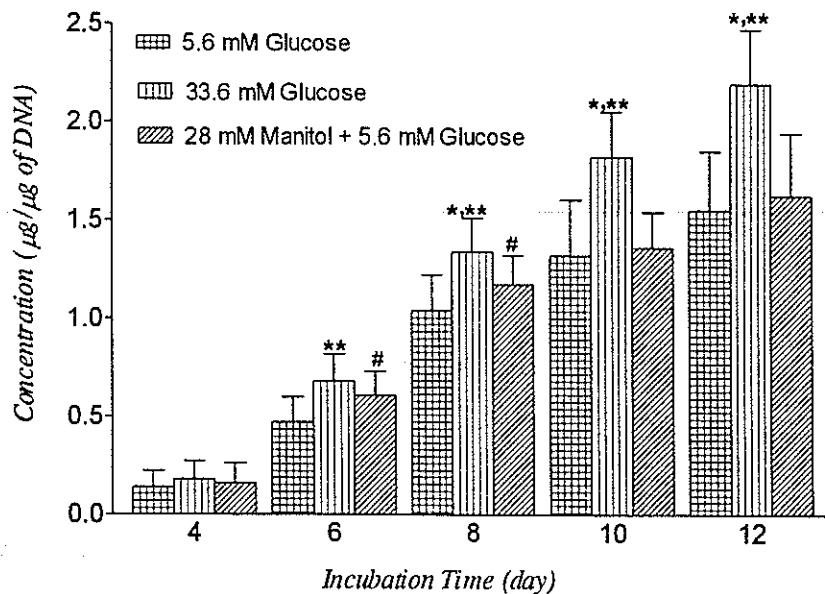
Figure 3. Effect of High Glucose Concentration (33.6 mM) on Fibronectin Level Secreted into Cell Culture Supernatant by Human Glomerular Mesangial Cells. #P<0.05: As compared to normal glucose (5.6 mM) group. *P<0.05, **P<0.01: As compared to manitol (5.6mM glucose + 28 mM manitol) and normal glucose (5.6 mM) groups, respectively. Each bar represents the mean ± SEM of 7 flasks.



matrix expansion occurs early in diabetes and correlates with the severity of the disease (2,6). Since mesangial matrix expansion may result from increased synthesis and/or decreased degradation of ECM glycoproteins such as FN and COL, the effects of high glucose concentrations on human glomerular MCs in culture were studied. Our results showed that, in the presence of high glucose, the MCs exhibit decreased proliferation and increased concentration of the ECM proteins, FN and COL in the cell culture supernatants. The

decrease in the MC proliferation was not due to the hypertonicity of glucose, since this effect was not observed with similar osmotic concentrations of manitol, and this suggest that glucose may have a suppressive effect on MC proliferation independent of osmolality. The present results show that the effect of glucose on the cell proliferation is concentration-dependent (see figures 1 and 2). The mechanism by which glucose inhibits cellular proliferation is not clear, but there are reports on the involvement of numerous factors including TGFβ (7), cAMP (8), atrial

Figure 4. Effect of High Glucose Concentration (33.6 mM) on Type IV Collagen Level Secreted into Cell Culture Supernatant by Human Glomerular Mesangial Cells. #P<0.05: As compared to normal glucose (5.6 mM) group. *P<0.05, **P<0.01: As compared to manitol (5.6mM glucose + 28 mM manitol) and normal glucose (5.6 mM) groups, respectively. Each bar represents the mean ± SEM of 7 flasks.



natriuretic factor (9), heparin (10) and prostanoids (11) have been shown to inhibit the MC mitogenesis. The effect of glucose on these mediators is largely unknown but TGF β gene expression has shown to be up regulated in cultured proximal tubular cells exposed to high glucose levels (12). Some investigators have also shown the elevation of glomerular TGF β in human (13-14) and experimental animals (13, 15-17) diabetic nephropathy. Moreover, the production of metabolic waste products unique to a high glucose environment may contribute to the observed decrease in cellular proliferation. Our results also indicate that MCs exposed to high glucose concentration increased FN level in the cell culture supernatants. The mechanism by which elevated glucose levels may stimulate mesangial cell FN synthesis and/or decreased its degradation is not clear. However, FN gene expression is known to be up regulated by high glucose levels through a cAMP response element (18) and some cytokines including epidermal growth factor (EGF) (19), gamma interferon (20), platelet-derived growth factor (PDGF) (19), interleukin-6 (IL-6) (21), TGF β (7,18) and heparin (22). The present results demonstrate that increased FN and COL concentrations in human mesangial cell culture supernatants, induced by high glucose are also seen with media rendered hypertonic with mannitol, suggesting that the

increase in FN and COL concentrations associated with high glucose may, in part, be mediated by increased osmolality. Fibronectin synthesis may also be regulated by feed back inhibition through the interaction of matrix proteins with cell surface receptors (23-24). Glucose may abrogate normal feed back mechanism that leads to an increase in FN synthesis. In vitro studies have demonstrated the ability of TGF β to increase the synthesis by mesangial and epithelial cells of various ECM components, including proteoglycans (25-26), type I and IV collagens (26-28) and FN (27). Therefore, one possible mechanism by which high glucose levels increase the levels of FN and COL in the supernatant by MCs may be through the increase synthesis of TGF β by these cells which in turn not only increase the synthesis of ECM components including FN and COL but also decrease the degradation of the ECM by decreasing in the activity of plasminogen activator/plasmin/matrix metalloproteinase 2 (MMP-2) cascade (29). Finally, it is concluded that human glomerular MCs may increase synthesis and/or decrease degradation of FN and COL in response to hyperglycemia and this response could contribute to the accumulation of ECM, which is common to diabetic glomerulosclerosis.

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