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PROTECTIVE EFFECTS OF ANTIOXIDANTS ON HIGH GLUCOSE-INDUCED MALFUNCTIONS IN HUMAN GLOMERULAR MESANGIAL CELLS.

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ABSTRACT

Altered functions of mesangial cells induced by high glucose concentrations are thought to play an important role in the pathogenesis of diabetic nephropathy. We therefore investigated the effect of high glucose (39.2 mM) alone and in combination with taurine (500 μM) or vitamin E (100 μM) in serum free medium (RPMI 1640) on the proliferative growth response and turnover of type IV collagen by human glomerular mesangial cells (GMC). The results showed that the high glucose level decreases the proliferation of the GMC which is reversed by taurine and vitamin E. In order to control the osmotic effects of high glucose, the GMC were also cultured in the presence of manitol. Manitol had no effect on the proliferation of GMC. Furthermore, the results showed that addition of vitamin E or taurine to media containing high glucose could reverse and normalize the collagen turn-over by the cultured mesangial cells. These results suggest that taurine and vitamin E may function as endogenous agents in the kidney to limit the development of glomerulosclerosis in diabetic renal disease.

Key Words: Mesangial cells, Diabetic nephropathy, Antioxidants, High glucose and extracellular matrix.

INTRODUCTION

One of the major problems of diabetes is renal disease and the glomerular mesangial cells (GMC) have an important role in the pathogenesis of diabetic nephropathy (1, 2). Previous studies have indicated that expansion of mesangial matrix produced by GMC occurs early in diabetes and the degree of expansion due to accumulation of extracellular matrix (ECM) proteins correlates with the severity of diabetic renal disease (3). The major side effects of high glucose on the mesangial cells are inhibition of the cell growth, increases in the ECM proteins such as collagens and fibronectin and lipid peroxidation (4-6). Since the oxidative stress induced by high glucose concentration contributes to diabetic nephropathy (7-8), this study was designed to determine the effects of endogenous antioxidants such as vitamin E and taurine on high glucose-mediated injury to human glomerular mesangial cells.

MATERIALS & METHODS

Reagents. Amphotricin B, Penicillin, streptomycin, DNA (from Calf Thymus), type IV collagen and [^{3}H]-thymidine were purchased from Sigma Chemical Co. (St. Luis, MO). Fetal calf serum (FCS) and PBS were purchased from Biochrom KG (Berlin, Germany). The medium RPMI 1640 with L-glutamine was purchased from Biowhitttaer (MD, USA). Hoechst 33258 was purchased from Fluka (Deisenhofen, Germany).

Isolation and culture of glomerular mesangial cells. Human mesangial cells were isolated by a graded-sieve technique from primary glomerular explants of the nephrectomized patients with kidney tumor. 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 amphotricin B. For the outgrowth purpose, the mesangial cells were plated in 75-cm^2 flasks and incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. In the present study, passage number 6 to 10 of the mesangial cells were used.

Evaluation of the antioxidants effects on the cellular proliferation. To assess the effect of antioxidants on GMC proliferation, the cells were exposed to 5.6 mM or 39.2 mM glucose alone or in combination with vitamin E (100 μM)

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or taurine (500 μM) as antioxidants. For hypertonicity studies, 33.6 mM manitol + 5.6 mM glucose were used as control group for up to 6 days. To detect the amino acid specificity of taurine, the effect of glycine (1mM) on the proliferation and collagen production in the GMC were studied. The cellular proliferation was assessed by direct counting method using hemocytometer. In order to evaluate the cell growth by an alternative method, tritiated thymidine incorporation by the human GMC was also evaluated. For this purpose the groups were incubated for 6 days. The cells were subsequently pulsed with 1.0 μCi [^{3}H]-thymidine (5 Ci/mmol) and after eight hours, the cells were washed with PBS, trypsinized, transferred to fiberglass filters, and then radioactivity of each well was assayed in a scintillation counter.

Effects of the antioxidants on Type IV collagen (COL) concentration in tissue culture supernatant. To evaluate the effect of high glucose on COL concentration in the supernatant, the mesangial cells were cultured in 25 cm^2 flasks in media containing 5.6 mM and 39.2 mM glucose alone or in combination with vitamin E (100 μM) or taurine (500 μM) as well as in media containing 33.6 mM monitol + 5.6 mM glucose as control group for hypertonicity. All media were changed every 48 hours. On days 2, 4, and 6, COL concentration in the supernatants were measured using ELISA technique, and the results were expressed on the basis of DNA content of cells (μg/μg of DNA).

Assay of DNA. For the measurement 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

Effects of antioxidants on high glucose-induced antiproliferation in human glomerular mesangial cells. The effect of high glucose (39.2 mM) on mesangial cell growth was confirmed by a 29% reduction in the cell count ($P<0.01$) (figure 1). There was no significant effect of glucose, manitol, vitamin E, taurine or glycine on cell viability since in all experiments, over 95% of the cells excluded by the exclusion of trypan blue. Moreover, the results showed that exposure of the GMC to high glucose concentration (39.2 mM) led to a significant decrease in the cell proliferation in comparison with normal glucose (5.6 mM) on the basis of significant ($P<0.03$) reduction in [^{3}H]-thymidine incorporation (Figure 2). The inhibitory effect of elevated ambient glucose on GMC proliferation was not a consequence of hypertonicity of the medium, since the media containing 33.6 mM manitol + 5.6 mM glucose did not reproduce the effect of the high glucose concentration. As shown in figures 1 and 2, addition of vitamin E and taurine to high glucose media reversed the antiproliferative effect of an elevated ambient glucose level and restored significantly ($P<0.05$) the incorporation of [^{3}H]-thymidine to normal. Vitamin E was slightly more effective than taurine in promotion of the mesangial cell growth in high glucose media in the thymidine uptake assay. Neither taurine nor vitamin E had any effect on the GMC proliferation in media containing a normal ambient glucose level. Glycine (1 mM) alone did not change GMC growth in media containing ambient normal glucose and it did not prevent the antiproliferative effect of high glucose concentration.

Effects of antioxidants on increased extracellular type IV collagen (COL) by high glucose concentration in human glomerular mesangial cells. To assess effects of vitamin E (100 μM) or taurine (500 μM) as antioxidants on COL turnover by GMC in the presence of high glucose, the GMC were exposed up to 6 days to 5.6 mM or 39.2 mM glucose in the presence or absence of vitamin E or taurine. The results are shown in fig 3 in which each data point represents total COL concentration accumulated by the GMC. Because total COL concentration in the supernatant would vary with cell number, the results were expressed on the basis of DNA content (μg/μg of DNA). As shown in the figure 3, high glucose concentration (39.2 mM) could significantly ($P<0.01$) increase concentration of COL into the supernatants. To evaluate the effects of hypertonicity due to the high glucose concentration on the COL level, the effect of control group [manitol (33.6 mM) + glucose (5.6 mM)] on this glycoprotein level in the cell culture supernatants was studied. As shown in figure

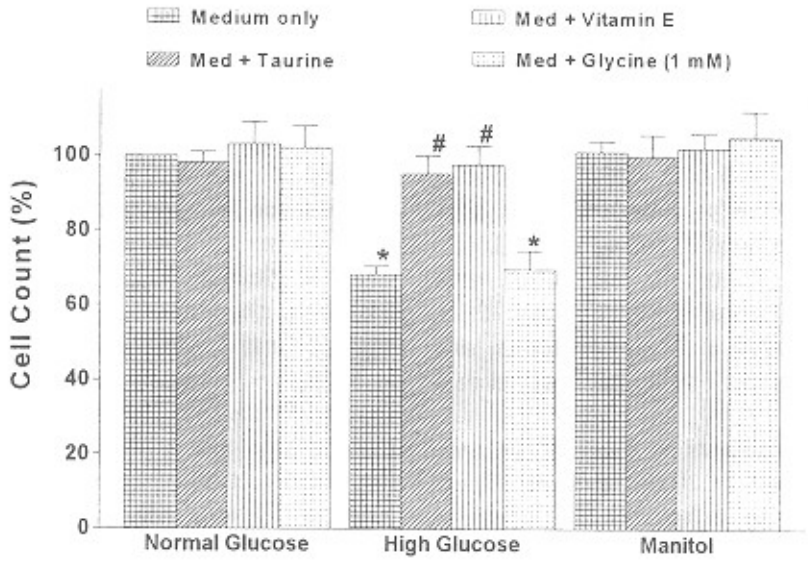

Figure 1. Antioxidants Effects on the Mesangial Cells Growth as Evaluated by Direct Counting Method. *P<0.01: As compared to normal (5.6 mM) glucose with no additives. #P<0.05: As compared to high glucose (39.2 mM) with no additives. The cultures were incubated for 6 days. Each bar represents the mean ± SEM of 8 flasks.

3, the control group as compared to normal glucose 95.6 mm) could increase COL concentration significantly (P<0.05). However, addition of vitamin E (100 μM) or taurine (500 μM) to the media containing high glucose reversed and normalized extracellular COL turnover by the cultured GMC. These results indicate that vitamin E and taurine may limit the development of renal disease in diabetic patients.

DISCUSSION

Diabetic glomerulosclerosis is characterized by the accumulation of extracellular matrix (ECM) proteins in the mesangium (3). Previous studies have demonstrated that the degree of mesangial matrix expansion occurs early in diabetes and correlates with the severity of the disease (1,3). Since mesangial matrix expansion may result from increased cellular proliferation, as well as increased synthesis and/or decreased degradation of ECM glycoproteins such as collagens and fibronectin, the effects of high glucose in combi-nation with the antioxidants (vitamin E and taurine) on human GMC were studied. The pre-sent results similar to those of previous study (5) showed that in the presence of high glucose, the GMC exhibit decreased proliferation and increaseed concentration of the ECM protein, COL in the cell culture supernatants. The decrease in the GMC proliferation was not due to the hypertonicity of glucose, since this effect was not observed with similar osmotic concentration of manitol. It is concluded that glucose may have a suppressive effect on GMC proliferation independent of osmolality. The results showed that antioxidants could reverse these effects of high glucose on GMC. Our previous study showed that the effect of glucose on the cell proliferation is concentration-dependent (5). The mechanism, by which glucose inhibits cellular proliferation and the influence of antioxidants to reverse this effect remaines unknown. Numerous factors including TGFß (9), cAMP (10), atrial naturetic factor (11), heparin (12),

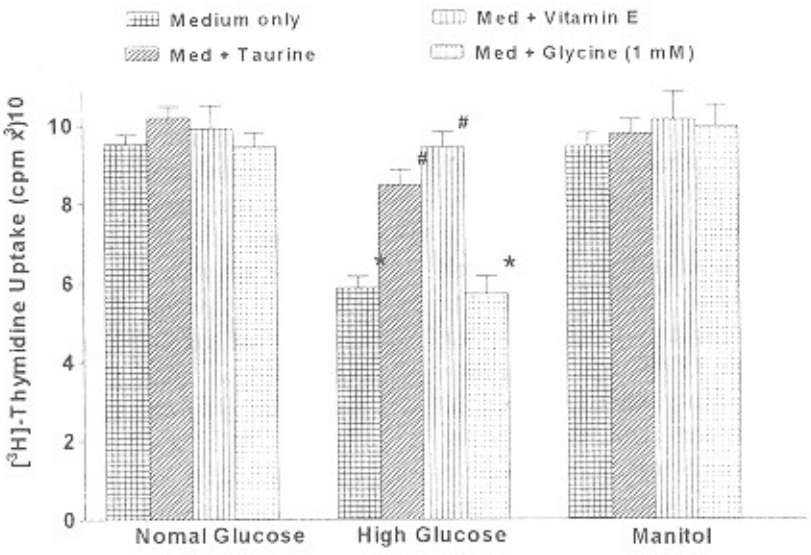

Figure 2. Antioxidants Effects on the Mesangial Cells proliferation, Evaluated by Tritiated Thymidine Incorporation Method. *P<0.03: As compared to all groups of normal glucose (5.6 mM). #P<0.05: As compared to medium containing high glucose (39.2 mM) only. The cultures were incubated for 6 days. Each bar represents the mean ± SEM of 8 flasks.

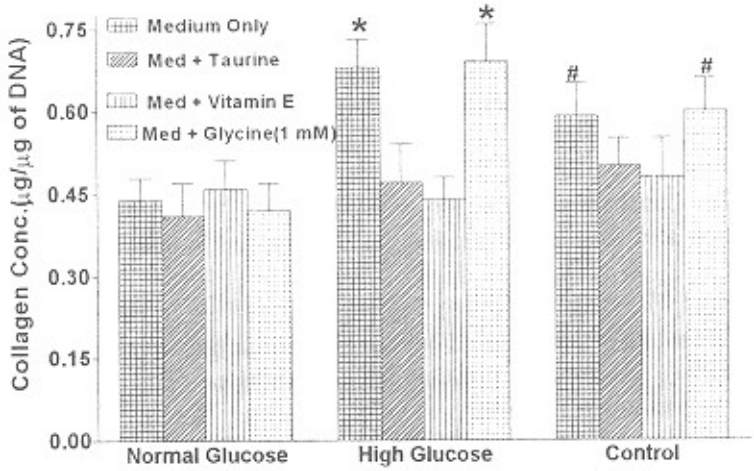

Figure 3. Effects of High Glucose (39.2 mM) Alone and in Combination With Taurine (500 mM) or Vitamin E (100 mM) on Type IV Collagen Level Secreted into Cell Culture Supernatant by Human Glomerular Mesangial Cells. The control group contains 33.6 mM manitol + 5.6 mM glucose. #P<0.05, *P<0.01:As compared to normal glucose (5.6 mM) group. Each bar represents the mean ± SEM of 8 flasks. The cultures were incubated for 6 days.

warfarin (13) and prostanoids (14) have been shown to inhibit the GMC mitogenesis. The effect of glucose and antioxi-dants on these mediators is largely unknown but TGFß gene expression has been shown to be up regulated in cultured proximal tubular cells exposed to high glucose levels (15-16). Some investigations have also shown the elevation of glomerular TGFß in human (17-18) and experimental animals (17, 19-21) in 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 GMC exposed to high glucose concentration increased COL level in the cell culture supernatants. Otherwise glycine had no effect on COL turn-over. The protective effects of taurine on GMC are specific for this amino acid. The mechanism by which elevated glucose levels may stimulate mesangial cell COL synthesis and/or decrease its degradation is not clear. The present results demonstrated that increased COL concentration in human mesangial cell culture supernatants, induced by high glucose are also seen with media rendered hypertonic with manitol, suggesting that the increase in COL concentration associated with high glucose may be mediated in part by increased osmolality. *In vitro* and *in vivo* studies have demonstrated the ability of TGFß to increase the synthesis of various ECM components including proteoglycans (22-23), type I and IV collagens (23-26) and fibronectin (FN) (24) by mesangial and epithelial cells. Therefore, one possible mechanism by which high glucose levels increase the levels of COL in the supernatant by GMC may be through increased synthesis of TGFß by these cells which in turn not only increase the synthesis of ECM components including COL but also decrease the degradation of the ECM by decreasing in the activity of plasminogen activator/plasmin/matrix metallo-proteinase 2 (MMP-2) cascade (27). Some reports have shown that oxidized low density lipoprotein (Ox-LDL) can induce alpha 1 (I), alpha 1 (III) and alpha (IV) collagen mRNA expression in human GMC, where vitamin E and antibody against Ox-LDL can cause a marked reduction in collagen mRNA stimulated by LDL (28). Furthermore, taurine can block increases in type I and IV collagen expression in mesangial cells in response to TGF-β1 (26). Therefore, it is supposed that vitamin E and taurine may inhibit the oxidative stress induced by high glucose through glucose auto-oxidation and oxidation of LDL to oxidized-LDL (Ox-LDL) by GMC. Moreover, the endogenous anti-oxidants (vitamin E and taurine) may protect mesangial cells against diabetic injury, in part by modulation of the gene expression and/or synthesis of various mediators including TGF-β.

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