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The Effects of High Glucose Concentration on Angiotensin II- or Transforming Growth Factor-β-Induced DNA Synthesis, Hypertrophy and Collagen Synthesis in Cultured Rat Mesangial Cells

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Hyperglycemia is a principal characteristic of diabetes, and has an influence on many cellular functions. In order to investigate whether the intracellular signaling pathways inducing proliferation, hypertrophy and matrix synthesis of mesangial cells are altered in a diabetic environment, we evaluated the effects of a high concentration of extracellular glucose(25 mM; 450 mg/dl) on [^{3}H]thymidine uptake, hypertrophy, and [^{3}H]proline incorporation into a collagenase-sensitive protein, induced by angiotensin II(Ang II) or transforming growth factor(TGF)-β, in cultured rat mesangial cells. The exposure to a high glucose concentration for 7 days significantly inhibited Ang II(10^{-6} M)-induced [^{3}H]thymidine uptake, compared to normal glucose concentration(5 mM)(M±S.D., 1050±100 cpm/well vs 550±97, $p<0.05$), and markedly prevented the inhibition of [^{3}H]thymidine uptake by TGF-β(1 ng/ml)(132±10 vs 340±67, $p<0.05$). The administration of H-7(50 μM), a protein kinase C(PKC) inhibitor, did not reverse these effects of high glucose on [^{3}H]thymidine uptake. On flow cytometric analysis of cell size, the mean cell size was significantly greater for the cells exposed to high glucose or treated with Ang II or TGF-β, compared to that for the untreated cells. But the addition of Ang II or TGF-β to the cells exposed to high glucose did not show further enlargement in size. The exposure to high glucose and the treatment with Ang II or TGF-β significantly increased collagen synthesis, measured by [^{3}H]proline incorporation. The Ang II- or TGF-β-induced increase of [^{3}H]proline incorporation did not show changes under high glucose culture condition, compared to normal glucose concentration(Ang II, 27880±3560 cpm vs 26978±2284, TGF-β, 26559±3700 vs 25800±1660, $p>0.05$). In conclusion, although the signaling pathway for DNA synthesis by Ang II or TGF-β are influenced, possibly mediated by PKC-independent mechanism(s), the pathway inducing hypertrophy or collagen synthesis by both agents appears to be unchanged under the high extracellular glucose concentration in cultured rat mesangial cells.

Key Words: High glucose, angiotensin, TGF-β, mesangial cells

Diabetic nephropathy is increasingly becoming the most important cause of end-stage renal disease. The disease is characterized by hypertrophy of glomerular elements, thickening of the basement membrane, progressive accumulation of extracellular matrix components in the mesangium, and tubulointerstitial fibrosis(Lane *et al.* 1990; Adler, 1994). Hyperglycemia per se appears to be an essential factor that is necessary for the development of diabetic nephropathy. Elevated glucose concentration in a culture medium stimulates the biosynthesis of collagen and other

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matrix constituents(Ayo *et al.* 1991b; Haneda *et al.* 1991), and modulates cell growth such as that resulting in the development of tubular hypertrophy (Wolf *et al.* 1991). In general, such effects of high glucose concentration may arise as a consequence of non-enzymatic glycation (Silbiger *et al.* 1993), disordered myoinositol metabolism (Guzman *et al.* 1991), increased de novo synthesis of diacylglycerol and activation of protein kinase C (PKC) (Ayo *et al.* 1991a), or modulations of the response to hormones and growth factors including transforming growth factor (TGF)-β (Mene *et al.* 1993).

Mesangial cells have been reported to express renin-like enzyme activity and generate angiotensin I (Ihm *et al.* 1994). Angiotensin II (Ang II) induces proliferation, and stimulates the synthesis of extracellular matrix components such as fibronectin and collagen IV in cultured rat mesangial cells (Wolf and Neilson, 1993). Ang II increases the level of the TGF-β mRNA and the production of active TGF-β in rat mesangial cells. TGF-β, in turn, plays a major role in regulating the extracellular matrix turnover, through inhibiting matrix degradation, or increasing the synthesis of matrix protein receptors (Ignotz and Massague, 1986; Wolf and Neilson, 1993). TGF-β also inhibits mesangial cell proliferation and induces hypertrophy (Choi *et al.* 1993). These effects of Ang II and TGF-β are reminiscent of the findings of diabetic nephropathy. The inhibition of angiotensin-converting enzyme has been reported to ameliorate the extent of renal hypertrophy in diabetes (Lewis *et al.* 1993), and Yamamoto *et al.* reported the enhanced glomerular expression of TGF-β in experimental DM in the rat. These findings suggest that diabetic nephropathy could be partly mediated by Ang II and TGF-β (Yamamoto *et al.* 1993).

While the reported effects of Ang II or TGF-β on mesangial cells have been studied in the presence of normal glucose concentrations, the responses to these growth factors under the glucose concentrations in the range occurring in diabetes are not well known. Considering the activation of PKC by elevated glucose levels, changes in the signal transduction system following Ang II or TGF-β stimulation can be expected. In order to elucidate the role of hyperglycemia on the process of diabetic nephropathy, we evaluated the influences of high glucose concentrations on DNA synthesis, hypertrophy, and collagen synthesis in response to Ang II or TGF-β in cultured rat mesangial cells. Moreover, we also tested whether the influences of high glucose could be modified by a PKC inhibitor.

MATERIALS AND METHODS

Reagents

RPMI-1640 without glucose, D-glucose, mannitol, collagenase, trichloroacetic acid, Ang II, TGF-β, and H-7 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Fetal calf serum and trypsin-EDTA were purchased from Gibco Laboratories (Grand Island, NY, USA).

Mesangial cell culture

Primary cultures of rat mesangial cells were prepared according to the methods of Choi *et al.* (1995). Briefly, the renal cortices from 6 to 8 Sprague Dawley rats were collected, and passed through successive stainless steel sieves of 200, 150, and 75 μm pore size. The isolated glomeruli were plated on culture dishes in RPMI-1640 containing 20% fetal calf serum (FCS), penicillin, streptomycin, and insulin. Morphological examination confirmed a homogenous population of stellate-shaped cells typical of mesangial cell. The cells were capable of growth in D-valine substituted medium and were negative with antibodies (DAKO Japan Co., Kyoto, Japan) to anti-desmin and factor VIII by immunoperoxidase staining. Cell passages 3 to 15 were used in these studies.

Effects of high glucose on Ang II- or TGF-β-induced DNA synthesis, hypertrophy and collagen synthesis

Equal numbers of mesangial cells were seeded into culture wells, and after 24 hours, the media was changed to either media containing

5 mM of glucose (normal glucose) or media supplemented with glucose to a final concentration of 25 mM(high glucose). After a 7 days exposure to high glucose, DNA synthesis was evaluated by [^{3}H]thymidine incorporation as described below. To determine the effects of high glucose concentration on the response to Ang II (10^{-6} M) or TGF-β (1 ng/ml), cells preincubated in normal or high glucose for 6 days were exposed to both agents for 24 hours. Hypertrophy was evaluated after exposure to high glucose for 10 days, and cells were always maintained in the same concentration of glucose. Collagen synthesis was assayed after exposure to high glucose for 7 days, and after incubation in high glucose for 5 days, the influence of high glucose on responses to both agents were assessed following a 48-hour exposure to both agents under the same glucose concentration. To investigate whether the effects of high glucose was mediated via PKC activation, H-7, a PKC inhibitor, was tried for the same period for Ang II or TGF-β stimulation. H-7 was dissolved in distilled water and was diluted to a final concentration of 50 μM with incubation medium. The same concentration of mannitol was used as an osmotic control.

[^{3}H]thymidine incorporation

Cells were subcultured in flat-bottomed 96-well culture plates at a density of 1×10^4 cells per well. At confluency, the cells were synchronized into a quiescent state by incubation in 0.5% FCS media, and were exposed to Ang II or TGF-β for 24 hours. During the last 6 hours of culture, they were pulsed with 1 μCi/well of [^{3}H]-thymidine (Amersham, Arlington Heights, IL, USA). The cells were collected with a cell harvester (Titertek Cell harvester 550, Flow Laboratories, Irvine, Scotland, UK) onto glass microfiber filter paper. The radioactivity was assayed by counting the filters in scintillation fluid for β-emissions. Cellular incorporation of [^{3}H]thymidine was used as an index of DNA synthesis.

Assay for cell hypertrophy

Cell size was determined by the methods used by Choi *et al.* with minor modifications (Choi *et al.* 1993). Mesangial cells were incubated in media containing 20% FCS, and exposed to high glucose concentration for 10 days. On day 11, cells were washed three times with phosphate buffered saline, detached from the plates with trypsin/EDTA, and titrated to obtain a single cell suspension at a density of 10^6 cells/ml in PBS containing 2% FCS and same glucose concentration. The cells were then subjected, without fixation, to flow cytometry with an FACStar plus (Becton-Dickinson, Mountain View, CA, USA) analysis. Histograms of a minimum of 10 events were generated and gated from the forward-angle light-scatter profile, providing an index of cell size. Relative cell size for 5000 cells in each sample was determined by quantification of forward light scattering.

[^{3}H]proline incorporation

De novo collagen synthesis was measured by the incorporation of [^{3}H]-proline into collagenase-digestible material as described by Ihm *et al.* (1994). The cells were plated at 1×10^5 cells per well in 6-well plates in basal medium supplemented with 20% FCS, 50 μg/ml each of sodium ascorbate and β-aminopropionitrile, and the indicated amounts of various materials according to the experimental design as mentioned above. The cells were labeled with 5 μCi of [^{3}H]-proline (Amersham, Arlington Heights, IL, USA) for 24 hours. After an additional 24 hours, proteins in the cells and the media were precipitated with 2 ml of 10% TCA and 1% tannic acid. The precipitated material was centrifuged at 4℃ and 100×g for 10 minutes. The material was washed three times with fresh 6% TCA and then dissolved in 2 ml of 0.2 N NaOH. A portion from each sample was subjected to liquid scintillation counting. The remainder was adjusted to contain NaCl 100 mM, Hepes 50 mM, $CaCl_3$ 3 mM, and pH 7.4. Bacterial collagenase type III 100 U/ml was then added, followed by incubation for 12 hours at room temperature. Following collagenase digestion the proteins were again precipitated, washed with ice-cold TCA, solubilized in NaOH and subjected to liquid scintillation counting. Col-

lagenase-sensitive [^{3}H]proline incorporation was defined as the difference between the counts before and after the collagenase digestion. On the average, collagenase-sensitive [^{3}H]proline incorporation represented about 50% of the total proline incorporation.

Statistics

Data were expressed as mean±S.D.. ANOVA combined with Scheffe's test was performed to compare multiple groups, and statistical significance was defined as $P<0.05$.

RESULTS

Effects of high glucose concentration on Ang II- or TGF-β-induced [^{3}H]thymidine uptake

Compared to cells grown in normal glucose concentration, the exposure of mesangial cells to high glucose concentration for 7 days significantly inhibited [^{3}H]thymidine uptake, suggesting inhibition of DNA synthesis (677±75 cpm/well vs 500±83, $p<0.05$, Fig. 1). In normal glucose concentration, Ang II at a concentration of 1×10^{-6} M markedly stimulated [^{3}H]thymidine uptake, compared to untreated cells (677±75 vs 1050±100, $p<0.05$, Fig. 1). On the other hand, treatment with TGF-β inhibited [^{3}H]thymidine uptake in a dose-dependent manner, and 1.0 ng TGF-β significantly decreased [^{3}H]thymidine uptake, compared to the untreated cells(624±40 vs 270±20, $p<0.05$, Fig. 2).

Concerning the effect of high glucose concentration on Ang II-induced [^{3}H]thymidine uptake, the exposure to high glucose for 48 hours significantly inhibited the increase of [^{3}H]thymidine uptake stimulated by Ang II, compared to Ang II-stimulated cells in normal glucose concentration (1050±100 vs 550±97, $p<0.05$, Fig. 1). On the other hand, under the high glucose culture condition, [^{3}H]thymidine uptake of cells treated with TGF-β was significantly higher than the level of cells under normal glucose, although the level was lower than the untreated control cells (340±67 vs 132±10, $p<0.05$, Fig. 1). These results suggest

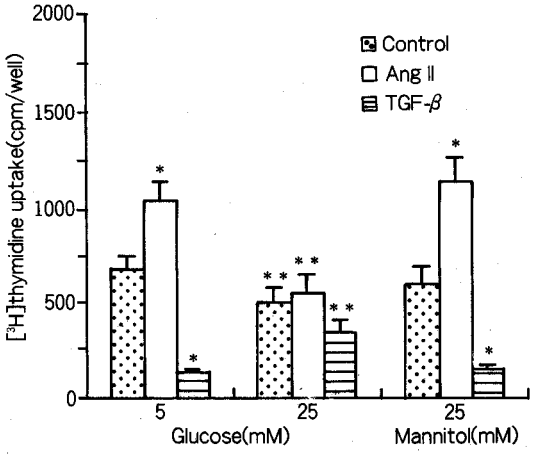

Fig. 1. *Effects of high glucose concentration on [^{3}H]thymidine uptake of mesangial cells treated with angiotensin II(Ang II) and transforming growth factor-β(TGF-β). The exposure to high glucose concentration inhibited 1×10^{-6} M Ang II-stimulated-[^{3}H]thymidine uptake, whereas 1.0 ng/ml TGF-β-induced inhibition of [^{3}H]thymidine uptake was partially recovered. *: $p<0.05$ vs control, **: $p<0.05$ vs 5 mM glucose, N=3 in triplicate.*

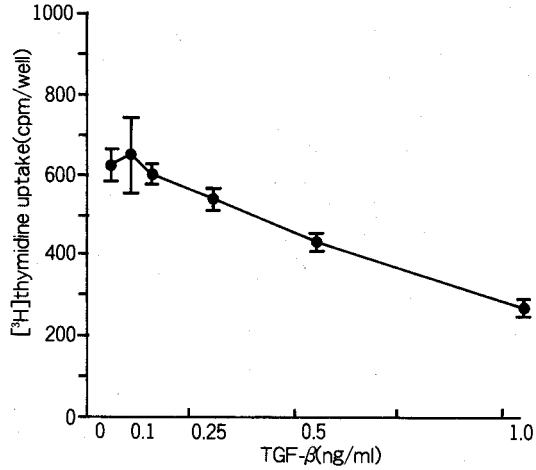

Fig. 2. *Dose-response curve of TGF-β-induced inhibition of [^{3}H]thymidine uptake in mesangial cells. TGF-β inhibited [^{3}H]thymidine uptake in a concentration-dependent fashion. N=2 in triplicate.*

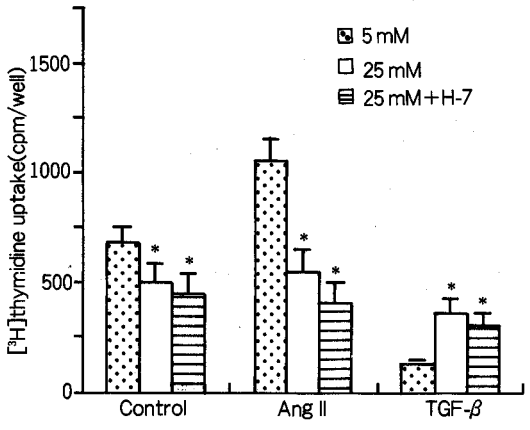

Fig. 3. *Effects of protein kinase C antagonist, H-7 on [³H]thymidine uptake following Ang II and TGF-β stimulation in the presence of 25 mM glucose. The addition of 50 μM H-7 did not influence the effects of high glucose concentration on [³H]thymidine uptake following Ang II and TGF-β stimulation. *: $p<0.05$ vs 5 mM, N =3 in triplicate.*

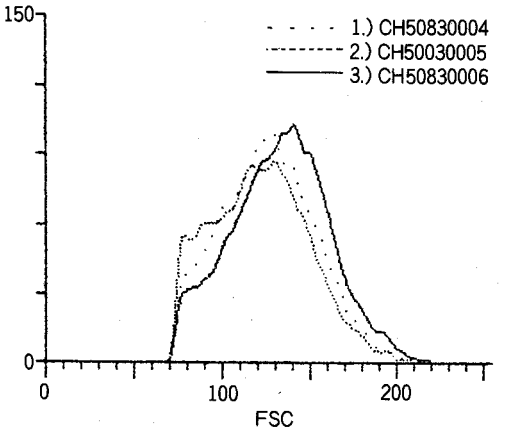

Fig. 4. *FACS analysis of mesangial cells incubated in normal and high glucose concentrations. The peak of the curve corresponded to the mean size of cells. The solid line, and dotted lines with small and large intervals represent the cells cultured under the 25 mM, 5 mM of glucose and 25 mM of mannitol for 10 days, respectively. The mean cell size was greater in cells exposed to 25 mM glucose concentration, compared to 5 mM glucose or 25 mM mannitol. One experiment is shown.*

that the TGF-β-induced inhibition of [³H]thymidine uptake was partially recovered under high glucose environment. Our osmotic control, the cells exposed to 25 mM mannitol, did not show a difference of [³H]thymidine uptake from normal glucose, suggesting that the effects of high glucose on [³H]thymidine uptake are not due to the osmolar effects of glucose(Fig. 1).

Regarding the possibility of mediating high glucose-induced changes of [³H]thymidine uptake by PKC, the concurrent addition of 50 μM of H-7 for 24 hours did not prevent the inhibition of Ang II-induced [³H]thymidine uptake under high glucose concentration (550±97 vs 400 ±100, $p>0.05$, Fig. 3). Following the addition of H-7, the recovery from the TGF-β-induced inhibition of [³H]thymidine uptake was also not changed(355±70 vs 300±55, $p>0.05$, Fig. 3)

Effects of high glucose concentration on Ang II- or TGF-β-induced hypertrophy

Fig. 4 displays the FACS analysis of mesangial cells incubated in a high glucose culture condition. Forward cell scatter on the x-axis was considered as a measurement for cell diame-

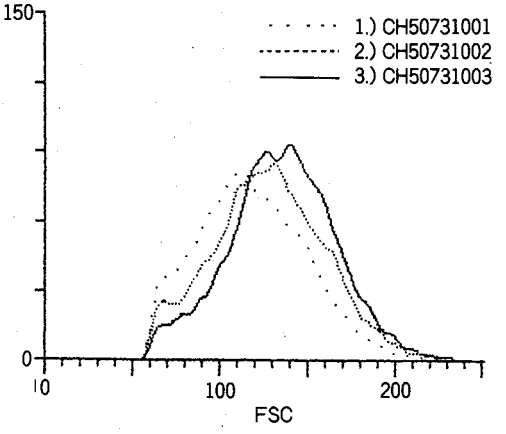

Fig. 5. *FACS analysis of mesangial cells following treatment with Ang II and TGF-β. The solid line, and dotted lines with small and large intervals represent the cells treated with 1.0 ng/ml of TGF-β, 1×10^{-6} M of Ang II, and control under 5 mM of glucose, respectively. The treatment with TGF-β or Ang II for 48 hours increased the cell size, compared to untreated control cells.*

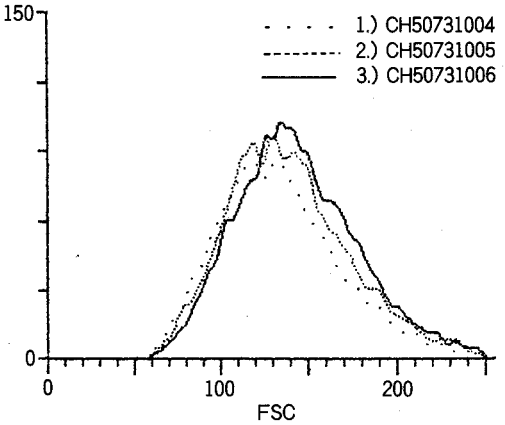

Fig. 6. *Effects of a high glucose concentration on mesangial cell hypertrophy in response to Ang II and TGF-β. The solid line, and dotted lines with small and large intervals represent the cells treated with TGF-β, and Ang II at same concentrations as in Fig. 5, and untreated control under 25 mM of glucose for 10 days, respectively. The exposure to high glucose concentration did not augment the increase in cell size following Ang II or TGF-β stimulation.*

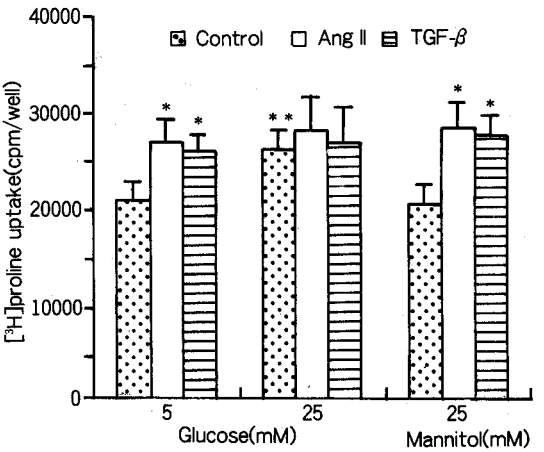

Fig. 7. *Effects of high glucose concentration on [³H] proline uptake of mesangial cells treated with Ang II and TGF-β. The addition of 1×10^{-6} M Ang II or 1.0 ng/ml TGF-β stimulated [³H] proline uptake significantly in 5 mM glucose, compared to untreated cells. The exposure to high glucose concentration did not have an influence on Ang II- and TGF-β-stimulated-[³H] proline uptake. *: $p<0.05$ vs control, **: $p<0.05$ vs 5 mM glucose, N=3 in triplicate*

Table 1. Effects of protein kinase C antagonist, H-7 on mesangial cell hypertrophy in response to Ang II and TGF-β, in the presence of 25 mM glucose

	Changes in cell diameter(unit¹)	
	5 mM	25 mM
Control	105±11	125±13*
Ang II	121±10*	128±15*
TGF-β	129±12*	131±11*

1: Forward light scater, arbitrary unit
*: $p<0.05$, vs control in 5 mM glucose, N=3

ter. A shift to the right of the curve indicates an increase in cell diameter. The mean cell size was significantly greater in mesangial cells grown in the presence of high glucose, compared to cells grown at a normal glucose concentration (Fig. 4). The mean forward light scattering as a measurement of cell diameter, averaged 125±13 and 105±11 units ($p<0.05$), respectively. Treatment with 1×10^{-6} M Ang II or 1.0 ng/ml TGF-β also significantly increased the cell size, compared to the untreated cells (Ang II: 121±10 units, TGF-β: 129±12, vs 105±11, $p<0.05$, Table 1, Fig. 5). Concerning the effect of high glucose concentration on Ang II- and TGF-β-induced hypertrophy, the mean size of the cells treated with Ang II or TGF-β under a high glucose concentration was not significantly different from the Ang II- or TGF-β-treated cells under normal glucose (Table 1, Fig. 6). On comparison between the cells exposed to high glucose alone and the cells treated with Ang II or TGF-β, the increase of cell size was not statistically significant.

Effects of high glucose concentration on Ang II- or TGF-β-induced [³H]proline uptake

As shown in Fig. 7, there was a significant increase of collagen synthesis, reflected by [³H]proline uptake, in mesangial cells exposed

to high glucose concentration for 7 days, compared to cells cultured in normal glucose concentration (21030±1750 cpm/well vs 25890±2040, $p<0.05$, Fig. 7). Compared to untreated cells, [^{3}H]proline uptakes were also stimulated by Ang II at a concentration 1×10^{-6} M, and TGF-β at a concentration of 1.0 ng/ml for 48 hours (21030±1750 vs 26978±2284 in Ang II, 25800±1660 in TGF-β, $p<0.05$, Fig. 7). In mesangial cells exposed to high glucose, Ang II- or TGF-β-induced [^{3}H]proline uptake was augmented to a similar degree compared to cells grown in the presence of normal glucose (Fig. 7). There was no statistically significant difference of Ang II- or TGF-β-induced [^{3}H] proline uptake between the cells cultured in normal and high glucose concentrations.

DISCUSSION

The exposure to high glucose concentration for 7 days inhibited DNA synthesis of mesangial cells. Cosio has already reported the inhibitory effects of high glucose on DNA synthesis and cellular proliferations in human mesangial cells (Cosio, 1995). In rat mesangial cells, incubation with high glucose for more than 24 hours caused an increase in PKC levels (Ayo *et al.* 1991a). Glucose-induced elevations in PKC levels may be the explanation for the inhibition by high glucose. Cosio observed that PKC inhibition with Calphostin C prevented the inhibition of thymidine incorporation caused by 30 mM glucose, suggesting reversible inhibition of proliferation by high glucose through a PKC-dependent mechanism (Cosio, 1995). In addition, considering the relatively long exposure, protein glycosylation or the alteration of polyol metabolism could also be postulated upon as the inhibitory mechanisms (Guzman *et al.* 1991; Silbiger *et al.* 1993).

The stimulation of DNA synthesis by Ang II was inhibited by the incubation with high glucose concentrations, whereas the inhibition of DNA synthesis by TGF-β was reversed under a high glucose culture condition. Ang II stimulates DNA synthesis through phospholipase C (PLC), and the activation of PLC induces PKC activation and a rise in the cytosolic calcium concentration (Wolf and Neilson, 1993). From these findings, the possibility of influencing the stimulatory pathways of DNA synthesis through PLC system by high glucose can be speculated. The desensitization of Ang II receptors caused by PKC activation under high glucose culture condition can be one explanation (Mene *et al.* 1993). The negative effects of high concentration of glucose on proliferative response are demonstrable even under culture conditions associated with a stimulatory effect of Ang II on DNA synthesis.

It has been suggested that TGF-β may function as a cellular switch, tending to stimulate or inhibit proliferation and other cell functions depending on the basal state of the cell (Kaname *et al.* 1992). It therefore is tempting to speculate that TGF-β may act to suppress proliferation of mesangial cells under conditions when these cells are stimulated to proliferate, as might occur in the presence of an acute proliferative GN, whereas under conditions in which the mesangial cell growth is suppressed the cells could be stimulated to grow (Wolf *et al.* 1992b). Under high glucose conditions, as in the suppressed state, the stimulation of DNA synthesis by TGF-β can be expected. In our experiment, the inhibitory effect on DNA synthesis by TGF-β was partially recovered, as expected. TGF-β has been shown to inhibit DNA synthesis by an effect that may be related to dephosphorylation of the retinoblastoma gene product (Ziyadeh and Sharma, 1995), and this inhibitory effect appears to be prevented under high glucose culture conditions.

In our experiment, the possibility of mediating the effects of high glucose on Ang II- and TGF-β-induced DNA synthesis by PKC activation was evaluated. Although the exposure of high glucose activates PKC activity, the addition of H-7, an PKC inhibitor, did not prevent the antiproliferative effects of glucose. This finding suggests that the incubation with high glucose inhibits DNA synthesis by a PKC-independent mechanism. It has also been postulated that the pathways distal to PKC could be influenced under high glucose envi-

ronments. But, this can not be confirmed from our study, in which the thymidine incorporation per well has been evaluated. Further studies on the changes of DNA content in individual cells are required.

Ziyadeh *et al.* reported high glucose-induced hypertrophy of proximal tubular cells, demonstrating the effect of high ambient glucose concentrations on tubular cell growth (Ziyadeh *et al.* 1990). As the early lesions affecting the glomeruli in diabetics, glomerular hypertrophy is also a component of diabetic renal hypertrophy (Adler, 1994). In our experiment, the exposure to 25 mM glucose for 7 days induced significant increase in cell diameter, suggesting hypertrophy. These results are consistent with reports on cultured proximal tubular cells by Wolf *et al.* (1991).

Cellular hypertrophy involves not only a modest increase in cell diameter, but also a change in cellular volume or mass. Increases in cell dimensions demonstrated by FACS, did not differentiate between osmotic swelling and hypertrophy-related increases in structural mass (Ziyadeh *et al.* 1990; Wolf *et al.* 1991). This growth stimulatory effect of high glucose was independent of the osmolarity of the media, since increasing the osmolarity of normal glucose media with mannitol did not induce an increase in cell diameter. In addition, considering the higher rate of collagen synthesis in high glucose, the increase of cell dimensions in our study seems to be due to increased cellular mass rather than osmotic swelling, reflecting hypertrophy (Ihm *et al.* 1994). The present study provides an evidence that high glucose can induce cellular hypertrophy in cultured mesangial cells as measured by an increase in cell size.

Ang II is capable of inducing hypertrophy in vascular smooth muscle cells (Wolf and Neilson, 1993). The concomitant addition of a dose of 10^{-6} M Ang II did not show further increase in cell size in a high glucose condition. As the potential mechanisms, decreased receptor density under high glucose concentrations or changes of signal transduction following Ang II stimulation can be suggested (Williams *et al.* 1992). Wolf *et al.* have previously shown that Ang II-induced hypertrophy can be blocked by pertussis toxin and agents increasing intracellular cAMP, suggesting signal transduction by pertussis toxin-sensitive inhibitory G-proteins, and down-regulation of adenylate cyclase (Wolf *et al.* 1991). Cells grown for a prolonged time in high glucose may not be able to lower their intracellular cAMP after Ang II stimulation. A higher level of intracellular cAMP is a feature of diabetic cells (Wolf *et al.* 1991). Ang II also activates the sodium-hydrogen antiporter in proximal tubular cells, leading to an increase of cell sodium concentration (Liu and Cogan, 1988). It remains to be demonstrated whether cellular hypertrophy is mediated by this regulatory pathway in mesangial cells.

Rat mesangial cell hypertrophy in response to TGF-β has been reported by Choi *et al.* (1993). Like the results from the Ang II treatment, there was no further increase of cell dimension in high glucose medium. The regulatory mechanisms of TGF-β are complicated. TGF-β negatively regulates the expression of its own receptor at the level of transcription or mRNA stability (Wrana *et al.* 1992; Choi *et al.* 1993). In addition, the regulation of TGF-β activity may not reside solely in its level of synthesis, but also at the level of activation/inactivation through the proteolytic release of the latency conferring proteins (Lyons *et al.* 1990). Although the mechanisms for the absence of further increase of cell should be clarified, we observed that Ang II or TGF-β treatment did not augment the enlargement of mesangial cells in the presence of high glucose.

In this study, overall collagen synthesis, as determined by [^{3}H]proline incorporation into collagenase-sensitive proteins, was significantly increased after 7 days of exposure of the cells to high glucose concentrations. This finding is consistent with the changes which occur in diabetic nephropathy such as the accumulation of collagen, and mesangial expansion (Lane *et al.* 1990; Adler, 1994). Because activation of PKC can stimulate fibronectin synthesis, glucose-induced changes in PKC may explain the increased collagen synthesis (Ayo *et al.* 1991b; Studer *et al.* 1995).

Ang II induces an increase in the expression

and synthesis of fibronectin in a dose- and time-dependent manner (Wolf *et al.* 1992a; Egido, 1996). Rat mesangial cells produce large amounts of collagen IV when stimulated by Ang II (Wolf *et al.* 1992a). Ang II stimulates the production and secretion of active TGF-β_1, which in turn stimulates the synthesis and deposition of matrix components (Egido, 1996). In the present study, the addition of Ang II in the presence of high glucose did not cause further increase of collagen synthesis. From this result, it is likely that the pathway increasing collagen matrix synthesis by Ang II is not altered under high glucose conditions. This finding also partly supports the absence of further increase of hypertrophic response to concurrent treatment with Ang II and high glucose.

TGF-β stimulates the synthesis of a number of extracellular matrix proteins, such as fibronectin and collagen, by a variety of cells, including mesangial cells (Ignotz and Massague, 1986; Kaname *et al.* 1992; Wrana *et al.* 1992). It also inhibits matrix degradation by both increasing the activity of protease inhibitors and decreasing proteases (Mackay *et al.* 1989; Choi *et al.* 1993), and stimulates the synthesis of matrix protein receptors such as integrins (Kreisberg *et al.* 1994). We tested whether the addition of TGF-β can further increase collagen synthesis under high glucose condition. Like with the Ang II treatment, no further increase of collagen synthesis was seen. This finding suggests that the signal transduction pathway of stimulating collagen synthesis by TGF-β is not influenced by the exposure to high glucose. Studer *et al.* reported that PKC signals induced fibronectin synthesis and TGF-β bioactivity in mesangial cells (Studer *et al.* 1995). In this regard, under a high glucose culture condition, where the activation of PKC has been reported, changes in signaling pathways inducing collagen synthesis by TGF-β was expected. But there were no changes of collagen synthesis in the presence of high glucose, compared to normal glucose. Possibly, PKC signals do not play a major role in TGF-β-induced collagen synthesis.

In conclusion, although the signaling pathway for DNA synthesis by Ang II or TGF-β are influenced, possibly mediated by PKC-independent mechanism (s), the pathway inducing hypertrophy or collagen synthesis by both agents appears to be unchanged under the high extracellular glucose concentration. The possibility of mediation of diabetic nephropathy by Ang II and TGF-β can be suggested.

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