High glucose-induced intercellular adhesion molecule-1 (ICAM-1) expression through an osmotic effect in rat mesangial cells is PKC-NF- κ B-dependent

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Abstracts

Aims/hypothesis. Infiltration of mononuclear cells and glomerular enlargement accompanied by glomerular cell proliferation are very early characteristics of the pathophysiology of diabetes. To clarify the mechanism of early diabetic nephropathy, we measured [³H]-thymidine incorporation and cell numbers to show the influence of a high ambient glucose concentration and the osmotic effect on rat mesangial cell proliferation. We also measured the effect of high glucose on the expression of intercellular adhesion molecule-1 and vascular adhesion molecule-1 by flow cytometry and semiquantitative RT-PCR in mesangial cells and the adhesion of leukocytes to mesangial cells.

Methods/results. Cells exposed to high D-glucose (30 mmol/l) caused an increase in [³H]-thymidine incorporation and cell numbers at 24 and 48 h and normalized at 72 h ($p < 0.05$), whereas these changes were not found in high mannitol (30 mmol/l), IL-1 β , or TNF α -stimulated mesangial cells. Cells exposed to high-glucose (15, 30, or 60 mmol/l) or osmotic agents (L-glucose, raffinose and mannitol) showed that intercellular adhesion molecule-1 expression began to increase after 24 h, reached its maximum at 24 and 48 h and gradually decreased afterwards. The stimulatory effects of high glucose and high mannitol on mRNA expression were observed as early as 6 h and reached its maximum at 12 h. Up-regulation of

ICAM-1 protein and mRNA was also found in IL-1- β and TNF- α -stimulated mesangial cells. Neither vascular adhesion molecule-1 protein nor mRNA expression was, however, affected by high glucose and high mannitol. Notably, the protein kinase C inhibitors calphostin C and staurosporine reduced high glucose- or high mannitol-induced intercellular adhesion molecule-1 mRNA expression and high glucose-induced proliferation. Furthermore, the NF- κ B inhibitor *N*-tosyl-L-phenylalanine chloromethyl ketone reduced high glucose- or high mannitol-induced intercellular adhesion molecule-1 mRNA expression and high glucose-induced proliferation. Results showed that high glucose (15, 30 mmol/l) or high concentrations of osmotic agents remarkably increased the number of adherent leukocytes to mesangial cells ($p < 0.01$) compared with control cells (5 mmol/l D-glucose). Functional blocking of intercellular adhesion molecule-1 on mesangial cells with rat intercellular adhesion molecule-1 monoclonal antibody, calphostin C, staurosporine, or *N*-tosyl-L-phenylalanine chloromethyl ketone significantly inhibited high glucose- or high mannitol-induced increase in leukocyte adhesion ($p < 0.05$).

Conclusion/interpretation. These results suggest that high glucose can upregulate intercellular adhesion molecule-1 protein and mRNA expression but not vascular adhesion molecule-1 expression in mesangial cells and promote leukocyte adhesion through

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Abbreviations: ECM, Extracellular matrix; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular adhesion molecule-1; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; PKC, protein kinase C; NF- κ B, nuclear factor-kappa B; FBS, fetal bovine serum; NG, normal glucose; HG, high glucose; HM, high mannitol.

up-regulation of intercellular adhesion molecule-1 through osmotic effect, possibly depending on the protein kinase C nuclear factor-kappa B (PKC-NF- κ B) pathway. High glucose itself can also promote mesangial cell proliferation through the PKC-NF- κ B pathways. We conclude that hyperglycaemia in itself

seems to be an important factor in the development of early diabetic nephropathy. [Diabetologia (2000) 43: 1544–1553]

Keywords Diabetes mellitus, glucose, ICAM-1, mesangial cells, PKC-NF- κ B.

Diabetic nephropathy is characterized by the early appearance of hypertrophy of both glomerular and tubular elements associated with proteinuria and decreased glomerular filtration and the eventual progressive accumulation of extracellular matrix (ECM) components in the glomerular mesangium and tubulointerstitium, all leading to glomerulosclerosis and tubulointerstitial fibrosis [1–3].

Recent clinical trials have shown that the degree of hyperglycaemia is an important predictor of diabetic renal complications [4]. Results of *in vitro* studies have given supportive evidence that increased ambient glucose concentrations exert an important influence on ECM metabolism that generally stimulates the synthesis and inhibits the degradation of the ECM [3]. The effect of hyperglycaemia on mesangial cell proliferation, however, is still controversial [5, 6]. A recent study has shown that a high glucose concentration in the culture medium results in transient stimulation of mesangial cell proliferation and then in growth inhibition after 72 h [7]. These studies suggest that hyperglycaemia could play an important part in mesangial cell proliferation.

Evidence shows that mononuclear cells, including monocytes and macrophages, play an important part, not only in inflammatory glomerular and interstitial disease but also in renal disease not related to active glomerulonephritis, especially diabetic nephropathy [8, 9]. Accumulated and activated monocytes or macrophages or both synthesize a variety of growth factors and cytokines, including IL-1, TNF- α , TGF- β , platelet-derived growth factor (PDGF), and angiotensin II. Such factors could contribute to the expansion of the ECM and glomerulosclerosis. Some investigators have shown that macrophages or monocytes or both are more abundant within glomeruli in moderate and advanced glomerulosclerosis than in mild diabetic glomerulosclerosis and normal glomeruli from control subjects [8].

During the process of glomerulosclerosis and atherogenesis, adhesion of circulating monocytes to mesangial cells and endothelial cells is thought to be an early event, mediated by interaction of adhesion molecules and their ligands. Among these adhesion molecules, E-selectin [10], intercellular adhesion molecule-1 (ICAM-1) [11], and vascular adhesion molecule-1 (VCAM-1) [12] are mainly responsible for monocyte adhesion to endothelial cells [13]. A recent study has reported that the expression of ICAM-

1 is up-regulated and promotes the recruitment of mononuclear cells in rat glomeruli through glomerular hyperfiltration during the early stage of diabetes [14]. Other recent studies have shown that increased adhesion of monocytes to human endothelial cells cultured under high osmotic conditions was closely associated with ICAM-1 expression in endothelial cells [15, 16]. In contrast, it has not been determined whether a high concentration of glucose can increase the expression of adhesion molecules such as ICAM-1 and VCAM-1 in cultured mesangial cells.

Several important consequences of protein kinase C (PKC) activation are relevant to diabetic nephropathy. In cultured renal cells, high concentrations of glucose activate PKC, which modulates gene expression of TGF- β and ECM protein and regulates proliferation of vascular cells [17, 18, 19]. It has been reported that induction of ICAM-1 expression is regulated by either activation of NF- κ B [20] under the influence of various cytokines or activated PKC by activation of TRE or AP-1 sites [21].

This study evaluates whether an increased ambient glucose concentration influences the proliferation of cultured rat mesangial cells. We investigate whether a high concentration of glucose can induce the expression of adhesion molecules, ICAM-1 and VCAM-1 and promote leucocyte adhesion to cultured rat MCs and we also examine the intracellular mechanism of induction of adhesion molecules.

Materials and methods

Materials. The RPMI 1640 (D-glucose 0 mmol/l), trypsin-EDTA solution, fetal bovine serum (FBS), D-mannitol and other tissue culture reagents were obtained from Sigma Chemical (St. Louis, Mo., USA). Human TNF- α and IL-1 β were purchased from R & D Systems (Minneapolis, Minn., USA). Calphostin C, genistein, staurosporine, and N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) were purchased from Sigma Chemical (St. Louis, Mo., USA). Rat monoclonal anti-ICAM-1 was obtained from Pharmingen (San Diego, Calif., USA). Rat monoclonal anti-VCAM-1 and pure goat anti-mouse monoclonal IgG antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif., USA). The cDNA probe for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Genosys Biotechnologies (Woodlands, Calif., USA). The [3 H]-Thymidine (7.4×10^4 KBq) was obtained from ICN Pharmaceuticals (Costa Mesa, Calif., USA). The PKC ELISA kit was purchased from Calbiochem (La Jolla, Calif., USA).

Cell culture. Rat mesangial cells (NMS2) were kindly provided by Dr. H. Abboud (Department of Internal Medicine, H.T.H.S.S.A., San Antonio, Tex., USA). Rat mesangial cells were cultured in RPMI-1640 containing 5 mmol/l D-glucose and 10% FBS with penicillin 100 units/ml and streptomycin 100 µg/ml at 37°C in an atmosphere containing 5% CO₂ with 95% humidity. Experiments were carried out in RPMI containing 0.2% FBS using cells between the 14th and 24th passages. At these passages mesangial cells showed normal morphology and function [5, 22]. To find the effect of a high D-glucose (HG) concentration on mesangial cell growth and expression of ICAM-1 and VCAM-1, cells were grown for different periods in 5 mmol/l D-glucose (normal glucose; NG), high glucose (HG) [15 mmol/l (15 HG), 30 mmol/l (30 HG), or 60 mmol/l (60 HG)], high L-glucose [L-HG; including 5 mmol/l glucose plus 10 (15 L-HG) or 25 mmol/l (30 L-HG) L-glucose], raffinose [RAF; including 5 mmol/l glucose plus 10 mmol/l (15 RAF) or 25 mmol/l (30 RAF) raffinose] and high mannitol [HM; including 5 mmol/l glucose plus 10 mmol/l (15 HM), 25 mmol/l (30 HM), or 55 mmol/l (60 HM) mannitol].

Cell viability was measured with trypan blue exclusion and lactate dehydrogenase assay (LDH; Sigma Chemicals, St. Louis, Mo., USA). These results showed they were not compromised after 4 days of incubation and various concentrations of medium used (data not shown).

Flow cytometry for ICAM-1 and VCAM-1. To investigate the effect of HG, HM and proinflammatory cytokines on rat mesangial cell expression of the ICAM-1 or VCAM-1 proteins, we used flow cytometry (FACS). The mesangial cells were cultured in media containing NG, HG, L-HG, RAF, HM, or, as positive controls, IL-1β and TNF-α. The mesangial cells were detached from culture flasks with trypsin, washed once with culture medium, and filtered through a 50-µl-pore polyamide gauze (Specrum Medical Industry, Calif., USA) to remove large cell aggregates. The number of viable mesangial cells was calculated, and the cells were washed with ice-cold PBS containing 0.5% bovine serum albumin (BSA; PBS/BSA). After washing cells with PBS, two million cells were incubated with 50 µl of the purified monoclonal antibody against ICAM-1 or VCAM-1 as a primary antibody (1:10 000 dilution) for 30 min at 4°C. Cells were washed twice with 1.0 ml ice-cold PBS/BSA, followed by 30 min incubation with 100 µl of fluorescein isothiocyanate-labelled monoclonal anti-mouse IgG antibody diluted 1:1000 in PBS/BSA at 4°C. The intensity of fluorescence was analysed using a FACS flow cytometer (Becton Dickinson, Mountain View, Calif., USA) at 488 nm.

Measurement of [³H]-thymidine incorporation into DNA and cell counts. The mesangial cells were transferred in 24-well culture plates at a density of 5 × 10⁴ cells per well. After reaching 80% confluency, these cells were maintained in a quiescent state in RPMI with 0.2% BSA for 48 h. The mesangial cells were then exposed to RPMI supplemented with NG; 15 HG, 30 HG, or 60 HG; 15 HM, 30 HM, or 60 HM; or TNF-α 20 µg/ml and IL-1β 20 µg/ml.

To examine the effects of PKC inhibitor (200 nmol/l calphostin C), tyrosine protein kinase inhibitor (50 µg/ml genistein), Ca⁺⁺-dependent protein kinase inhibitor (10 nmol/l staurosporine), NK-κB inhibitor (50 µmol/l TPCK), 30 HG, or 30 HM on [³H]-thymidine uptake, the intracellular signal inhibitors were applied 2 h before stimulation with high concentrations of glucose or mannitol. Next, mesangial cells were stimulated with HG or HM and then pulsed with 37 KBq of [³H]-thymidine (Amersham, Arlington Heights, Ill., USA) per well for the last 4 h. Cells were washed twice with ice-cold PBS and once with ice-cold 5% trichloroacetic acid (TCA) and

then incubated for 30 min at 4°C. After the TCA had been discarded, the cells were washed twice with ice-cold 0.25 N NaOH plus 0.1% sodium dodecyl sulphate. The cell lysate was neutralized with neutralizing buffer (1 mol/l TRIS:2.5 mol/l HCl 2:1). Half-millilitre aliquots were counted by liquid scintillation using a beta counter.

We also counted cell numbers using a standard hemocytometer.

Reverse transcription-polymerase chain reaction. Mesangial cells were exposed to RPMI containing 10% FBS supplemented with NG, 30 HG, or 30 HM for times ranging from 0 to 48 h. The mesangial cells were homogenised and total RNA was extracted with an RNase kit (Qiagen, Hilden, Germany). We used 5 µg of total RNA prepared from mesangial cells to synthesize cDNA. The reverse transcription reaction was carried out using a first-strand cDNA synthesis kit (Pharmacia Biotech, Tokyo, Japan) with a random primer supplied with the kit. The reaction mixture was incubated for 60 min at 37°C and heated for 5 min at 90°C in a thermal cycler (Takara Biomedicals, Tokyo, Japan). The primers used for the PCR were based on the sequence of rat cDNA in the GenBank database, and they were as follows:

ICAM-1: sense 5'-AGG TAT CCA TCC ATC CCA CA-3', antisense 5'-GCC ACA GTT CTC AAA GCA CA-3', expected 210 bp

VCAM-1: sense 5'-CTG ACC TGC TGC TCA AGT GAT GG-3', antisense 5'-GTG TCT CCC TCT TTG ACG CT-3', expected 260 bp

GAPDH: sense 5'-GCT ATG AGC CCT TCC ACG ATG C-3', antisense 5'-GAA TCT ACT GCG TCT TCA CC-3', expected 250 bp.

The PCR was done in a final volume of 5 µl containing 2 µl of each deoxyribonucleoside triphosphate (dNTP), 50 pmol/l of each primer, 2.5 U of *Taq* DNA polymerase and a buffer (45 mmol/l TRIS; 68 mmol/l KCl, 15 mmol/l dithiothreitol, 9 mmol/l MgCl₂, BSA 80 µg/ml). The polymerase chain reaction (PCR) was done using the following conditions: 94°C for 1 min, 54°C for 1 min, and 72°C for 2 min; 25 cycles for reduced glyceraldehyde-phosphate dehydrogenase (GAPDH), and 30 cycles for ICAM-1. Primers for GAPDH were used as the internal standard and for generation of a 250-bp fragment. A linear correlation was observed between the intensity of the bands and the number of PCR cycles (23, 25, and 27 cycles for GAPDH; 28, 30, and 32 cycles for ICAM-1) (data not shown). The PCR products were sequenced to confirm the identity of the PCR products using Qiagen DNA extraction kit (Qiagen, Chatsworth, Calif., USA) and an automatic sequencing machine (ABI Genetic Analyzer 310: PRISM, Branchburg Park, N.J., USA). Amplification products were separated by electrophoresis (2% agarose gel) and visualized by ethidium bromide staining. The bands on the positive film were scanned by densitometry (Model GT-8000; Epson, Tokyo, Japan).

Effect of calphostin C, genistein, staurosporine, and TPCK on ICAM-1 mRNA expression. To examine the effects of calphostin C, genistein, staurosporine, and TPCK on ICAM-1 mRNA expression in 30 HG or 30 HM media, mesangial cells were treated with these intracellular signal inhibitors 2 h before the addition of 30 HG or 30 HM for 24 h.

Leucocyte adhesion assay. To examine the effects of HG or HM on leucocyte adhesion on mesangial cells, mesangial cells were cultured for 24 h with HG (15 HG, 30 HG), L-HG (15 L-HG, 30 L-HG), RAF (15 RAF, 30 RAF), or HM (15 HM, 30 HM). For adhesion experiments we also used PKC inhibitor

(200 nmol/l calphostin C), tyrosine protein kinase inhibitor (50 µg/ml genistein), Ca⁺⁺-dependent protein kinase inhibitor (10 nmol/l staurosporine) or NK- κ B inhibitor (50 µmol/l TPCK) on 30 HG-induced or 30 HM-induced leucocyte adhesion to mesangial cells. The intracellular signal inhibitors were applied 2 h before stimulation with 30 HG or 30 HM. Leucocyte adhesion was assayed by modified methods from another study [23]. Briefly, confluent mesangial cell monolayers (1×10^5) on 12-mm coverslips were co-incubated for 30 min at room temperature under static conditions in 6-well cell culture plates with human leucocyte (1×10^5), in 1 ml of RPMI 1640 plus 1% FCS. In these assays, we used human leucocytes obtained from healthy male volunteers. To remove unattached leucocytes, each coverslip was washed by immersion three times in RPMI 1640 (Gibco BRL, Grand Island, N.Y., USA) plus 1% FCS, then once in PBS and fixed in 2% paraformaldehyde at 4°C. After staining with haematoxylin, the adherent leukocytes were counted in 10 selected at random high power fields ($\times 100$) on each of the coverslips and the results were expressed as the mean numbers of leucocytes for each field.

PKC measurement. To examine the effects of HG or HM on total PKC activity in mesangial cells, mesangial cells were cultured for 24 h with HG (15 HG, 30 HG) or HM (15 HM, 30 HM). The homogenate mesangial cell was centrifuged at low-speed ($1000 \times g$ for 10 min), medium-speed ($17000 \times g$ for 20 min) and high-speed spins ($100000 \times g$ for 1 h), respectively. The resulting pellet was used as membrane preparation. Total PKC activity in mesangial cells was determined by ELISA as described in the following. The PKC ELISA uses a synthetic PKC substrate and a monoclonal antibody that recognizes the phosphorylated form of the peptide. Briefly, in accordance to the protocol of the manufacturer, 108 µl of component mixture was added to each well of a polyvinyl plate and preincubated at 25°C for 5 min. The component mixture was prepared by combining the 13 µl $10 \times$ PKC reaction buffer, 13 µl 1 mmol/l adenosine triphosphate (ATP), 13 µl 500 µg/ml phosphatidylserine and 78 µl distilled water. The negative control for the assay contained an equal volume of EGTA instead of phosphatidylserine. A 12 µl kinase sample was added to each well and mixed. Then 100 µl of reaction mixture was transferred to each well and incubated 25°C for 10 min and 100 µl of 20% H₂SO₄ was added to each well. The plate was washed with wash solution five times. Biotinylated antibody 2B9 (100 µl) was added to each well and incubated at 25°C for 60 min. After five washes, 100 µl of peroxidase-conjugated streptavidin was added to each well and incubated at 25°C for 60 min. After additional washes, 100 µl of substrate solution was added to each well and incubated for 5 min. The reaction was stopped by adding 100 µl of 20% H₂SO₄ to each well. The optic density (OD) value was measured with microELISA reader (Bio-Rad, Hercules, Calif., USA) at 492-nm wavelength. The PKC activity for each sample was analysed in quadruplet and expressed as OD per milligram of protein.

Determination of translocation of NF- κ B. After mesangial cells were treated with the indicated concentration of D-glucose or mannitol for 24 h, nuclear fractions were prepared as described previously from 10^9 mesangial cells [24]. With nuclear fractions from each treatment, western blotting analysis was done using anti-p65 antibody (Santa Cruz, Calif., USA) and ECL system (Amersham Life Science, Buckinghamshire, UK).

Statistical analysis. Data are expressed as means $\pm$ SD. Differences between groups were examined for statistical significance using ANOVA with Bonferroni correction. A *p* value of

Fig. 1. Thymidine incorporation into rat mesangial cells incubated for 24 to 72 h in the presence of NG, 30 HG, 30 HM, or proinflammatory cytokines (IL-1 β and TNF- α). After 24 and 48 h, thymidine incorporation was stimulated by HG-containing medium compared with NG. *n* = 6. * *p* < 0.05 vs NG

less than 0.05 was considered a statistically significant difference. The average of the six experimental measurements was used for statistical analysis.

Results

Effect of high concentration of glucose and mannitol on rat MC proliferation. We investigated whether HG (30 HG), HM (30 HM), and the proinflammatory cytokines (IL-1 β and TNF- α) in serum-free medium have an effect on rat mesangial cell proliferation with various exposure times from 24 h to 72 h.

We found 30 HG at 24 and 48 h increased thymidine incorporation into DNA (*p* < 0.05) (Fig. 1). Similarly, the cell counts were increased compared with those of cells cultured in NG (30 HG at 24 h $5.26 \pm 0.77 \times 10^6$ cells/well, NG at 24 h 4.05×10^6 cells/well; *p* < 0.01; 30 HG at 48 h 5.58×10^6 cells/well, NG at 48 h 4.34×10^6 cells/well; *p* < 0.01). Of note, in 30 HG medium, thymidine incorporation and cell counts normalized to the basal level at 72 h. The increase in thymidine incorporation and the cell counts were biphasic at 30 HG, with the maximum response achieved at 48 h. In 15 HG and 60 HG cultured media, we also found the same responses to proliferation in the mesangial cell (data not shown). The stimulatory effect was not mediated by an increase in the osmolarity of the medium or proinflammatory cytokines (Fig. 1).

Effects of high concentrations of D-glucose, L-glucose, raffinose and mannitol on ICAM-1 expression on me-

Table 1. Effects of various exposure times and concentrations of D-glucose, mannitol, L-glucose and raffinose on ICAM-1 expression in rat mesangial cells

		12 h	24 h	48 h	72 h
NG	(5 mmol/l)	1	1	1	1
HG	15 mmol/l	1.06 ± 0.10	1.85 ± 0.52 ^a	1.59 ± 0.28 ^a	1.08 ± 0.13
	30 mmol/l	1.09 ± 0.11	2.56 ± 0.52 ^{ab}	1.65 ± 0.03 ^a	1.24 ± 0.10
	60 mmol/l	1.09 ± 0.11	2.59 ± 0.45 ^{ab}	1.91 ± 0.31 ^{ab}	1.00 ± 0.06
HM	15 mmol/l	0.92 ± 0.10	1.58 ± 0.21 ^a	1.66 ± 0.19 ^a	1.05 ± 0.07
	30 mmol/l	1.04 ± 0.12	2.64 ± 0.90 ^{ab}	2.14 ± 0.36 ^{ab}	0.99 ± 0.05
	60 mmol/l	1.11 ± 0.10	2.42 ± 0.44 ^a	2.07 ± 0.39 ^{ab}	1.04 ± 0.07
L-HG	15 mmol/l	1.01 ± 0.12	1.54 ± 0.33 ^a	1.52 ± 0.39 ^a	1.03 ± 0.22
	30 mmol/l	1.05 ± 0.12	2.77 ± 0.44 ^{ab}	2.44 ± 0.42 ^{ab}	1.11 ± 0.12
RAF	15 mmol/l	1.00 ± 0.09	1.63 ± 0.42 ^a	1.72 ± 0.52 ^a	1.03 ± 0.09
	30 mmol/l	1.08 ± 0.10	2.81 ± 0.66 ^{ab}	1.99 ± 0.23 ^{ab}	1.09 ± 0.04
IL-1 β		1.01 ± 0.02	3.27 ± 1.33 ^a	1.77 ± 0.05 ^a	0.84 ± 0.11
TNF- α		1.04 ± 0.04	2.78 ± 1.07 ^a	2.08 ± 0.29	1.00 ± 0.08

The relative amount of ICAM-1 is expressed as the ratio of the amount of protein to that found during growth NG at various times (12, 24, 48, and 72 h). The results are expressed as mean $\pm$ SD ($n = 6$). Abbreviations; NG, normal D-glucose;

HG, high D-glucose; L-HG, high L-glucose; HM, high mannitol; RAF, raffinose

^a $p < 0.05$ vs NG at 12 h and various times

^b $p < 0.05$ vs 15 HG or 15 HM

sangial cells. In the basal state (NG), ICAM-1 protein was expressed at low but detectable levels. Both HG and other high concentrations of osmotic agents (L-HG, RAF and HM) induced a dose-dependent increase in the surface expression of ICAM-1, which reached its plateau at 60 and 30 mmol/l, respectively. Exposure of the cells to 60 HG or 30 HM resulted in a 2.6 ± 0.5 -fold and 2.6 ± 0.9 -fold increase, respectively, compared with those cultured in the NG in the expression of ICAM-1 (Table 1). Time-dependent increases of ICAM-1 expression induced by HG, L-HG, RAF and HM were also shown and seemingly peaked at 24 h. In addition, in all cultured media, ICAM-1 expression was decreased to the basal levels at 72 h. The same results were obtained with proinflammatory cytokines (Table 1). The VCAM-1 expression, however, was not detectable under NG conditions and was not induced by HG and other osmotic agents (data not shown).

These findings suggest that ICAM-1 is the main adhesion molecule on rat mesangial cells induced by HG and that the changes of ICAM-1 expression induced by HG or other osmotic agents could be the stimulatory effect mediated by an increase in the osmolarity.

Effects of high concentrations of glucose and mannitol on ICAM-1 mRNA expression. We evaluated the expression of ICAM-1 mRNA with a cDNA probe detecting a transcript of 209 bp and compared it with GAPDH mRNA for semiquantitative measurement. In NG medium, a small amount of ICAM-1 mRNA was constitutionally expressed from 0 h to the end of stimulation, 48 h (Fig. 2). Of note, NG-induced ICAM-1 mRNA expression was 2.5 ± 0.4 -fold greater at 12 h than at 0 h. Exposure of the cells to 30

HG or 30 HM induced a time-dependent increase in ICAM-1 mRNA (Fig. 2). Exposure to 30 HG or 30 HM increased ICAM-1 mRNA by 3.0 ± 0.3 -fold after 24 h and 5.1 ± 0.5 -fold after 12 h compared with 0 h; and this response persisted for 48 h. This effect was, however, not present after an incubation time of only 4 h. The increase in ICAM-1 mRNA was dose-dependent for glucose and mannitol (Fig. 3), with the maximum responses achieved at 30 HG and 30 HM. These increases in ICAM-1 persisted for 48 h. The VCAM-1 mRNA expression, however, was not detected under NG conditions and there was no induction by HG and HM (data not shown).

Effects of calphostin C, staurosporine, genistein, and TPCK on response to high glucose- and mannitol-induced cell proliferation and ICAM-1 mRNA expression. Calphostin, staurosporine, and TPCK significantly ($p < 0.05$) inhibited proliferation of mesangial cells treated with 30 HG (Fig. 4). An inhibitory effect on proliferation was, however, not observed when mesangial cells were pretreated with genistein. We found a similar inhibitory effect on mesangial cell proliferation using direct cell counts (30 HG; $5.11 \pm 0.84 \times 10^6$ cells/well, calphostin; $4.45 \pm 1.12 \times 10^6$ cells/well; staurosporine $4.32 \pm 0.66 \times 10^6$ cells/well; TPCK 4.56×10^6 cells/well; respectively, $p < 0.01$).

We then examined whether PKC inhibitors and NF- κ B inhibitor influenced, likewise, the expression of ICAM-1 mRNA in mesangial cells pretreated with 30 HG or 30 HM (Fig. 5). Calphostin C, staurosporine and TPCK inhibited 30 HG-induced or 30 HM-induced ICAM-1 mRNA expressions ($p < 0.05$). These inhibitory effects were not found in genistein-pretreated mesangial cells.

Fig. 2A, B. Time-dependent expression of *ICAM-1* mRNA in response to 30 HG or 30 HM. **A** *ICAM-1* mRNA expression examined using RT-PCR. **B** Histogram represents means $\pm$ SD of the densitometric scans of the *ICAM-1* mRNA band from six experiments, normalized by comparison with *GAPDH* mRNA and expressed as the ratio at time 0. $n = 6$, * $p < 0.05$ vs 0 h; ** $p < 0.01$ vs 0 h. ■ NG, ▨ HG 30 mmol/l, □ 30 mmol/l

Leucocyte adhesion assay. Results showed that 15, 30 HG, 30 L-HG, 30 RAF or 15, 30 HM increased the number of adherent leucocytes to mesangial cells in respect to NG (Table 2, $p < 0.05$, respectively). Functional blocking of ICAM-1 on mesangial cells with rat monoclonal antibody ICAM-1, calphostin C, staurosporin or TPCK inhibited the increase in leucocyte adhesion induced by 30 HG, 30 L-HG, 30 RAF or 30 HM, respectively (Table 2, $p < 0.05$). When mesangial cells were pretreated with genistein such an inhibitory effect was not, however, observed.

Effects of high concentrations of glucose and mannitol on PKC and NF- κ B activities. Total PKC activity in mesangial cells was measured by ELISA as described above. Protein kinase C activity of 15, 30 HG or 15, 30 HM in the membrane fraction increased in respect to NG (Fig. 6, $p < 0.05$, respectively). In the cytoplasmic fraction, there were no differences in PKC activity

Fig. 3A, B. Dose-dependent expression of *ICAM-1* mRNA in response to NG, HG (15, 30, or 60 mmol/l), or HM (15, 30, or 60 mmol/l). The mesangial cells were incubated for 12 h with these stimuli. **A** *ICAM-1* mRNA expression examined using RT-PCR. **B** Histogram represents means $\pm$ SD of the densitometric scans of the *ICAM-1* mRNA band from six experiments, normalized by comparison with *GAPDH* mRNA and expressed as ratio of NG. $n = 6$, * $p < 0.05$ vs NG; ** $p < 0.01$ vs NG; *** $p < 0.05$ vs 15 HM

ty between the HG or HM and the control. Furthermore, to confirm whether NF- κ B activity using anti-p65 antibody was regulated by extracellular osmolarity, mesangial cells were incubated with various concentrations of HG and HM. The activity of NF- κ B increased significantly at 15, 30 HG and 15, 30 HM (Fig. 7). These results show that PKC-NF κ B can be activated by HG or HM and induced by an increase in extracellular osmolarity through osmotic effect.

Discussion

Hyperglycaemia in itself is an important factor in the development of early renal hypertrophy and mesangial cell proliferation in diabetes [7]. We showed that a high glucose concentration in the culture medium could promote biphasic mesangial cell proliferation. We also observed the up-regulation of cell-surface ICAM-1 protein and mRNA expression in rat mesangial cells and the increase in leucocyte adhesion on rat mesangial cells cultured in HG and HM media. In contrast, there was no change in the cell-surface

Fig. 4. Thymidine incorporation into mesangial cells incubated for 24 h in the presence of genistein (Gen), staurosporine (Sta), calphostin C (Cal) or *N*-tosyl-L-phenylalanine chloromethyl ketone (TPC) containing 30 HG or 30 HM. Means $\pm$ SD. $n = 6$. * $p < 0.05$ vs NG

expression of VCAM-1 on mesangial cells exposed to HG and HM conditions. Of note, these effects on ICAM-1 protein and gene activation and on leucocyte adhesion could be prevented by inhibitors of PKC and NF- κ B. Furthermore, mesangial cells from HG-media and HM-media showed similar cytosolic PKC activity to those from NG but higher membrane PKC activity. These results indicate that high concentrations of glucose and mannitol promote leucocyte adhesion on mesangial cells by up-regulation of ICAM-1 expression on the mesangial cell surface, possibly through the PKC-NF- κ B pathway.

Mesangial cells are known to express adhesion molecules and function as antigen-presenting cells (APCs) to circulating mononuclear leucocytes [25, 26], facilitating the migration of these cells into the glomeruli. Cell-surface adhesion molecules, especially ICAM-1 and VCAM-1, have a prominent role in inflammatory reactions characteristically seen in the

Fig. 5A, B. Effect of intracellular inhibitors, genistein, staurosporine, calphostin C, or TPCK, on expression of ICAM-1 mRNA. **A** ICAM-1 expression examined using RT-PCR. **B** Histogram represents means $\pm$ SD of the densitometric scans of the ICAM-1 mRNA band from six experiments, normalized by comparison with GAPDH mRNA and expressed as a ratio of NG. $n = 6$, * $p < 0.05$ vs NG

early phases of glomerular injury [23, 25–27]. We found that ICAM-1 was constitutively expressed on the surface of cultured mesangial cells and was more up-regulated by HG and HM. The VCAM-1 protein was, however, neither expressed constitutively nor affected by HG or HM. The effect of high concentrations of glucose on ICAM-1 expression was mimicked by exposing cells to medium containing high os-

Table 2. Effects of various exposure concentrations of D-glucose (NG, 15, 30, 60 HG), mannitol (15, 30 HM), L-glucose (30 L-HG) and raffinose (30 RAF) on leucocyte adhesion on

	NG	30 HG (15 HG)	30 HM (15 HM)	30 L-HG	30 RAF	IL-1 β	TNF- α
Anti-ICAM-1	45 $\pm$ 6	115 $\pm$ 16 ^a (106 $\pm$ 9 ^a)	133 $\pm$ 21 ^a (121 $\pm$ 11 ^a)	99 $\pm$ 10 ^a (90 $\pm$ 21 ^a)	113 $\pm$ 11 ^a (101 $\pm$ 21 ^a)	150 $\pm$ 21 ^a	128 $\pm$ 23 ^a
Genistein	–	62 $\pm$ 11	58 $\pm$ 13	57 $\pm$ 16	57 $\pm$ 16	–	–
Staurosporin	–	121 $\pm$ 11 ^a	141 $\pm$ 23 ^a	105 $\pm$ 15 ^a	115 $\pm$ 15 ^a	–	–
Calphostin	–	52 $\pm$ 9	49 $\pm$ 13	60 $\pm$ 15	51 $\pm$ 14	–	–
TPCK	–	53 $\pm$ 12	54 $\pm$ 10	53 $\pm$ 9	59 $\pm$ 15	–	–
	–	49 $\pm$ 13	50 $\pm$ 12	55 $\pm$ 13	53 $\pm$ 11	–	–

The results are expressed as means $\pm$ SD ($n = 6$). ^a $p < 0.05$ vs NG

mesangial cells. Mesangial cells were incubated with the indicated concentration of D-glucose, mannitol, L-glucose and raffinose

Fig. 6. Effect of HG (15, 30 mmol/l) or HM (15, 30 mmol/l) on total PKC activity in mesangial cells. Mesangial cells were incubated with NG, HG or 30 HM for 24 h. The cytosolic and membrane fractions were prepared as described in Materials and methods. Means $\pm$ SD. $n = 4$. * $p < 0.05$ vs NG. ■ NG; □ 15 HG; ▨ 30 HG; □ 15 HM; ▨ 30 HM

motric agents including L-glucose, raffinose and mannitol. Therefore, we can speculate that the up-regulation of ICAM-1 expression is mediated through an osmotic effect.

Compelling evidence has shown that infiltration of mononuclear cells is an early event in the glomeruli of patients with diabetes and of rats with streptozotocin-induced diabetes [8, 14]. A previous study [14] showed that the increased ICAM-1 expressions were paralleled by infiltration of monocytes or macrophages, or both, into diabetic rat glomeruli. In streptozotocin-induced diabetic rats, the numbers of glomerular macrophages/monocytes and degree of mesangial cellularity and mesangial matrix expansion were remarkably lower in the renal glomeruli of diabetic rats that had been irradiated with X-ray than in non-irradiated rats [28]. It was concluded that up-regulation of ICAM-1 expression in diabetic glomeruli is one mechanism that promotes the recruitment of mononuclear cells into diabetic glomeruli and that intraglomerular expression of ICAM-1 could be induced by glomerular hyperfiltration [14]. In contrast, we showed that increased ICAM-1 expression and leucocyte adhesion to mesangial cells could be induced by HG and other high concentrations of osmotic agents. It is also known that in the endothelial cells of diabetic rat glomeruli, increased ICAM-1 expression can be caused by plasma hyperosmolarity [15] and hyperglycaemia [16]. Therefore, in the murine mesangial cells, ICAM-1 expression associated with increased leucocyte adhesion to mesangial cells can be up-regulated by hyperglycaemia, at least in part by an osmotic effect.

Fig. 7. Effect of HG (15, 30, 60 mmol/l) or HM (15, 30 mmol/l) on NF- κ B activity in mesangial cells. Mesangial cells were incubated with NG, HG, or HM for 24 h. The nuclear extracts were prepared as described in Methods and Materials. With nuclear fractions from each treatment, western blotting was done using anti-p65 antibody

High ambient glucose concentrations in the culture medium in the absence of serum or any additional factors have a biphasic effect on the proliferation of a murine mesangial cell line [7]. Raising the ambient glucose concentration from 5.5 mmol/l to 25 mmol/l stimulates cell proliferation after 24 to 48 h but has a growth-inhibitory effect after 72 to 96 h. The proliferative effect is independent of the medium osmolarity. The late phase of glucose-induced growth inhibition is mediated by the bioactivation of endogenous TGF- β . We found the same biphasic effect of HG on mesangial cell proliferation. These changes were not induced by an osmotic agent; i.e., mannitol.

Of note, the biphasic response to HG or HM, with early stimulation and subsequent inhibition of thymidine incorporation, was reflected in similar changes in ICAM-1 expression. The enhancement of ICAM-1 protein and mRNA in mesangial cells was attenuated at times of longer than 48 h and 24 h, respectively. In addition, cell survival assay showed no statistically significant difference (data not shown). These findings are consistent with previous reports of death and apoptotic transformation of the cells related to changes of biphasic patterns in the time course of ICAM-1 expression in human umbilical vein endothelial cells (HUVEC) treated with glucose [29]. Those investigators suggested that exposure to HG for longer than 6 h might have a toxic effect on metabolism and attenuate the enhancement of ICAM-1 expression, even though it did not cause cell death and apoptosis. Therefore, in our study, a high concentration of glucose might have had two effects on mesangial cells; i.e., early enhancement of ICAM-1 expression and subsequent cell toxicity, which could diminish the expression of ICAM-1. The level of ICAM-1 expression in mesangial cells might be determined by both of these effects, as in HUVEC.

Little is known about the intracellular signals involved in triggering HG-induced and HM-induced ICAM-1 expression in mesangial cells. A previous study [30] suggested that PKC might be involved in IL-1, TNF- α , and lipopolysaccharide-induced up-regulation of ICAM-1 on endothelial cells. Evidence shows that the events that modulate adhesion and in-

filtration of leucocytes and up-regulation of ICAM-1 expression on HUVEC can occur through NF- κ B activation after exposure to HG medium [31]. We found that the functional blocking of ICAM-1 gene expression with PKC inhibitors (calphostin C), Ca⁺⁺-dependent protein kinase inhibitor (staurosporine), or a cytoplasmic inhibitor of NF- κ B (TPCK) in HG and HM reduced the up-regulation of ICAM-1 gene expression and leucocyte adhesion. We also observed that tyrosine protein kinase inhibitor (genistein) did not inhibit mesangial cell proliferation and reduce HG-induced and HM-induced up-regulation of ICAM-1 expression. Parallel to the ICAM-1 expression results, HG-induced or HM-induced leucocyte adhesion was not dependent on tyrosine protein kinase-mediated pathway. The recent evidence that PKC activation leads to dissociation of the NF- κ B/I κ B complex, permitting translocation to the nucleus and activation of its target genes [32], suggests the possibility of a link between PKC and NF- κ B activation in HG- and HM-induced up-regulation of adhesion molecule expression [31]. Other experimental evidence shows that expression of adhesion molecules is critically dependent on intracytoplasmic activation of NF- κ B. Thus, specific subunits of NF- κ B regulate expression of the E-selectin, ICAM-1, and VCAM-1 genes in endothelial cells activated by inflammatory cytokines [33–35]. We also showed increased membrane-associated PKC activity in mesangial cells cultured in the HG and HM. In accordance with these results, we hypothesize that up-regulation of ICAM-1 protein and gene expression in HG or HM medium in rat mesangial cells could activate the PKC–NF- κ B pathway. These changes might be related to the intracellular hyperosmolarity found in diabetes.

Finally, in this study we also found that the dissociation of their results regarding proliferation compared with ICAM-1 expression in the presence of HM medium. Thus, to further clarify the mechanism of the dissociation of hyperosmotic effect between on proliferation and on expression of ICAM-1, it might be important to confirm activation of other intracellular signal pathways, such as mitogen activated protein kinase (MAP) kinase-kinase and MAP kinase, through an osmotic mechanism in mesangial cells. In yeast system, some genes encoding MAP kinase and MAP kinase-kinase constitute osmosensing signal transduction pathway called high osmolarity glycerol (HOG) pathway and these genes are regulated by stress response element. Therefore, this HOG pathway could be a candidate mechanism for osmotic regulation of ICAM-1 expression in mesangial cells [15]. A recent study also showed that the activation of pleiotropic NF- κ B family of transcription factors might explain in part the diversity of growth factors and cytokine gene expression associated with endothelium [20]. Furthermore, hyperglycaemia induces a bewildering list of changes in vascular or renal cells

in animal models of diabetes or diabetic patients, because the flux of glucose and its metabolites are known to affect many cellular pathways [36]. It is likely that the common pathway, PKC–NF- κ B, by which all the intracellular and extracellular changes induced by hyperglycaemia are mediating their adverse effects is by altering various signal transduction pathways.

In conclusion, our results showed that: 1., a high concentration of glucose is a stimulator of rat mesangial cell proliferation; 2., culture in HG and HM medium up-regulates cell-surface ICAM-1 protein and mRNA expression, not expression of VCAM-1, on these cells; 3., HG- and HM-induced ICAM-1 expression showed biphasic patterns in the time course; 4., PKC and NF- κ B intracellular pathways can be activated and could be involved in up-regulated ICAM-1 gene expression in mesangial cells in high glucose and hyperosmolar conditions; 5., HG or other osmotic agents including HM considerably increased the number of adherent leucocytes to mesangial cells in respect to control. Furthermore, functional blocking of ICAM-1 on mesangial cells with rat ICAM-1 monoclonal antibodies, PKC or NF- κ B inhibitors inhibited HG- or hyperosmolar-induced increase in leucocyte adhesion. These findings suggest that hyperglycaemia in itself is an important factor in the development of early diabetic glomerular lesion. These findings might be relevant to understanding the nature of early glomerular lesions associated with increasing inflammatory cell recruitment in diabetic glomerulopathy.

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