Role of TGF-β/GLUT1 axis in susceptibility vs resistance to diabetic glomerulopathy in the Milan rat model

Carlo Ricci1, Carla Iacobini1,2, Giovanna Oddi1,2, Lorena Amadio1,2, Stefano Menini1, Maria Pia Rastaldi3, Aurora Frasheri1, Flavia Pricci2, Francesco Pugliese1 and Giuseppe Pugliese1

1Department of Clinical Sciences, ‘La Sapienza’ University, Rome, 2Department of Cell Biology and Neurosciences, Istituto Superiore di Sanità, Rome and 3Laboratory of Renal Immunopathology, San Carlo Borromeo Hospital, Milan, Italy

Abstract

Background. GLUT1 upregulation and increased glucose transport activity may contribute to extracellular matrix (ECM) accumulation characterizing diabetic nephropathy (DN). Rats of the Milan hypertensive strain (MHS) are resistant to both hypertensive and diabetic renal disease, due to a haemodynamic protection. On the contrary, those of the Milan normotensive strain (MNS) develop spontaneous glomerulosclerosis, and when rendered diabetic, show typical morphological and haemodynamic changes.

Methods. To assess whether susceptibility to diabetic glomerulopathy in MNS rats is associated with higher glucose transporter 1 (GLUT1) expression (and glucose transport activity) vs MHS rats, diabetic and nondiabetic MNS and MHS rats were followed for 6 months and mesangial cells derived from these animals were exposed to high glucose (HG) vs normal glucose (NG) conditions.

Results. Glomerular expression of GLUT1 protein and ECM and transforming growth factor-β (TGF-β) mRNA was significantly upregulated in diabetic vs nondiabetic MNS, but not MHS rats. Upon exposure to HG and/or TGF-β, mesangial cells from 1- and 8-month-old MNS rats showed higher glucose transport activity and GLUT1 membrane expression than those from age-matched MHS rats. Likewise, ECM and TGF-β production increased more markedly in response to HG and/or TGF-β in MNS vs MHS mesangial cells.

Conclusions. These data indicate that susceptibility to diabetic glomerulopathy in MNS rats is associated with increased GLUT1-dependent glucose transport activity in response to hyperglycaemia and/or TGF-β, which may amplify ECM overproduction. Conversely, the haemodynamic protection from glomerulosclerosis in MHS rats is associated with lack of upregulation of TGF-β/GLUT1 axis, thus supporting the concept that this axis may represent the link between haemodynamic and metabolic mechanisms of injury.

Keywords: diabetic nephropathy; extracellular matrix; glucose transport; GLUT1; renal haemodynamics; TGF-β

Introduction

The injurious effects of hyperglycaemia are characteristically observed in tissues which are not dependent on insulin for glucose entry into the cell and, hence, are not capable of down-regulating glucose transport along with elevation of extracellular sugar levels [1]. Indeed, glucose uptake was shown to increase in cultured mesangial cells upon exposure to high glucose (HG) containing media, via an upregulation of glucose transporter 1 (GLUT1) expression [2]. Moreover, treatment of mesangial cells with antisense GLUT1 blocked HG-induced GLUT1 and fibronectin overexpression [3]. Additionally, transfection of mesangial cells with GLUT1 cDNA mimicked the effects of hyperglycaemia at normal glucose (NG) concentrations, including the upregulation of extracellular matrix (ECM) production [4] and the activation of polyol pathway and protein kinase C (PKC) α and β1 [5,6]. Likewise, GLUT1 was found to be overexpressed in glomeruli from diabetic rats [7] and mice [8]. Furthermore, diabetic db/db mice carrying an antisense-GLUT1 transgene were protected against the development of diabetic nephropathy (DN) [8], whereas transgenic overexpression of GLUT1 driven by a
GULT1-mediated glucose transport and diabetic nephropathy

modified β-actin promoter in glomeruli of nondiabetic db/m mice produced features of DN, as indicated by the increased albuminuria and mesangial expansion [9].

In cultured mesangial cells, the prosclerotic cytokine transforming growth factor-β (TGF-β), known to play a pivotal role in the pathogenesis of DN [10], was shown to enhance GLUT1 expression and glucose transport activity, whereas the addition of neutralizing anti-TGF-β antibody prevented the stimulatory effect of HG on GLUT1 expression [11]. Thus, it was postulated that the overexpression of TGF-β induced by hyperglycaemia upregulates GLUT1 that, in turn, increases glucose uptake and, hence, further enhances the stimulation of TGF-β production, ultimately leading to mesangial ECM accumulation [12,13].

A recent report showed that, in addition to HG, stretching is also capable of activating the TGF-β/GLUT1 axis, which may represent the link between haemodynamic and metabolic mechanisms in glomerular injury associated with conditions in which systemic hypertension is transmitted to the glomerular micro-circulation [14]. In fact, TGF-β, GLUT1 and ECM were found to be upregulated in the Dahl salt-sensitive (Dahl-S) rat, characterized by glomerular hypertension and injury, but not in the young spontaneously hypertensive rat (SHR), when glomerular capillary pressure is still normal and renal disease has not yet developed [14]. Glomerular hypertension has been shown to occur also in diabetes, as a consequence of the loss of autoregulation due to afferent arteriole vasodilation [15]. Raised intraglomerular pressure is thought to play a pivotal role in the pathogenesis of DN, though the relative importance of haemodynamic vs metabolic factors has not been clarified yet [16].

In order to address this issue, we have utilized a unique animal model, the Milan rats, consisting of two genetically-related rat strains derived from a common Wistar ancestor, the Milan normotensive strain (MNS) and the Milan hypertensive strain (MHS). The MNS rats develop an age-dependent focal and segmental glomerulosclerosis, whose mechanisms are poorly understood, since classical risk factors cannot be identified [17,18]. The MHS rats develop a mild to moderate form of arterial hypertension [19], that has been attributed, at least partly, to a missense mutation of the α and β subunits of the adducin heterodimer, a protein participating in the assembly of spectrin–actin cytoskeleton [20]. This mutation is associated with altered cytoskeleton assembly, increased expression of Na⁺/K⁺ ATPase and upregulation of Na⁺/K⁺ pump activity of apical and basolateral membranes of renal tubules, with consequent sodium and water retention and increased blood pressure [21]. In contrast to the MNS, no glomerular disease occurs in MHS rats, despite the elevated blood pressure levels, possibly due to the marked hypertrophy of the intrarenal arteries protecting glomerular capillaries towards systemic hypertension and development of glomerulosclerosis [17,22].

Induction of diabetes in MNS rats resulted in typical lesions superimposed onto (and distinguished from) age-dependent glomerulosclerosis and associated with increased glomerular filtration rate (GFR) and filtration fraction. Diabetes also produced significant, though less marked changes in renal function and structure in progenitor Wistar rats. On the contrary, diabetic MHS rats did not show the characteristic changes in renal haemodynamics and remained free of renal disease, even after 6 months of uncontrolled diabetes, thus pointing to the importance of haemodynamic factors, which exert a permissive role towards hyperglycaemia-induced injury [23]. Conversely, in vitro, i.e. under conditions in which haemodynamics is not operating, mesangial cells isolated from both rat strains (before the onset of glomerulosclerosis and hypertension, respectively) responded to HG with an upregulation of TGF-β and ECM production. However, this response of ECM, but not TGF-β production was significantly higher in cells from MNS than in those from MHS rats [24]. This finding and the previous report of an age-dependent increase in ECM production and proliferative response to serum in mesangial cells from MNS, but not MHS rats [25] indicate a genetically determined hyperresponsiveness to sclerosing stimuli in the MNS rats that may underlie their susceptibility to spontaneous glomerulosclerosis.

This in vivo and in vitro study was aimed at verifying the hypothesis that the TGF-β/GLUT1 axis is activated in the glomerulosclerosis-prone MNS rats but not in the haemodynamically protected MHS rats, thus explaining their susceptibility and resistance, respectively, to DN and confirming that this axis represents the link between haemodynamic and metabolic mechanisms of injury.

Materials and methods

Design

In vivo studies. Adult (aged 12 weeks, weighing ~280g) male Milan rats of both strains (kindly provided by Prassis Research Institute sigma tau, Settimo Milanese, Milan, Italy) were divided into the following groups: time 0 control rats, that were killed immediately after initiating the study, and nondiabetic and diabetic MNS and MHS rats, that were sacrificed 3 and 6 months after diabetes induction (n = 6 per group). Rats were made diabetic by a single injection (via the caudal vein) of streptozotocin (Sigma Chemical Co., St Louis, MO), at a dose of 55 mg/kg body weight in citrate buffer (pH 4.5) [23]. The animals were housed and cared in keeping with the EC regulations and received water and food at libitum; when needed, diabetic animals were given supportive insulin treatment (Ultratard, Novo Nordisk, Denmark) to prevent ketosis without affecting significantly glycaemia. Need for treatment was defined depending on body weight measurements, as follows: in case of increased body weight, once a week injection of 4 IU/kg body weight; in case of unchanged body weight, twice a week injections of 4 IU/kg body weight; in case of decreased body weight, three times a week injections of 4 IU/kg body weight (or more). The mean weekly insulin dose was similar in the two diabetic groups. Metabolic control was monitored throughout the study by weekly insulin dose was similar in the two diabetic groups.
measuring blood glucose and body weight at regular intervals; blood pressure was also measured at time 0 and every 3 months thereafter. At time 0 (all nondiabetic rats, n = 18) and 3 months (3- and 6-month nondiabetic and diabetic rats, n = 12) and 6 months (only 6-month nondiabetic and diabetic rats, n = 6) after diabetes induction, rats were placed into metabolic cages to collect urines for the assessment of urine volume and proteinuria. Then, rats were anesthetized with i.p. ketamine (Imalgene®, 60 mg/kg body weight) and xylazine (Rompun®, 7.5 mg/kg body weight) and a blood sample was withdrawn for the assessment of glycated haemoglobin (Hb) and serum creatinine. The kidneys were quickly removed, cleaned of the surrounding fat, washed in sterile saline solution and weighed. A sagittal section of the right kidney was immediately fixed by immersion in phosphate buffered 4% paraformaldehyde solution and routinely embedded in paraffin for morphologic analysis. The remaining tissue was immerse in OCT and then frozen in isopentane-liquid nitrogen for measuring GLUT1 protein expression by immunofluorescence. Renal cortex from the left kidney was separated from medulla and used for glomeruli isolation by standard sieving techniques, followed by total RNA extraction for the assessment of ECM and TGF-β1 gene expression by reverse transcription polymerase chain reaction (RT-PCR).

In vitro studies. Glomerular mesangial cells were isolated from 1- and 8-month-old MNS and MHS rats (i.e. before and after the onset of glomerulosclerosis or hypertension, respectively) and characterized as previously described [24]. Cells between the third and the tenth passage were cultured for 10–15 days (over 2–3 passages) in Dulbecco-modified Eagle’s medium (Sigma, St Louis, MO) supplemented with 17% fetal bovine serum, 2 mmol/l glutamine and antibiotics (all obtained from Flow Laboratories, Irvine, Scotland, UK), but without insulin, at 37°C in 95% air and 5% CO2 humidified atmosphere, under NG (5.5 mmol/l) vs HG (30 mmol/l) conditions [24]. Then, monolayers were incubated for 18h in serum-free medium containing (a) vehicle or 25–200 pmol/l TGF-β (Calbiochem, San Diego, CA) ± the inhibitor of protein synthesis cycloheximide (5 μmol/l, Sigma), the blocker of sodium-dependent glucose transport phlorizin (10 mmol/l, Sigma) or the inhibitor of GLUT1-dependent glucose transport cytochalasin B (1 μmol/l, Sigma); and (b) β-TGF-β blocking antibody or control chicken IgG (30 μg/ml, R&D Systems, Minneapolis, MN). ECM and TGF-β1 mRNA levels and protein release were measured in conditioned media, GLUT1 protein expression and glucose transport were measured under these experimental conditions by RT-PCR ELISA western blot analysis and [3H]2-deoxyglucose ([3H]DOG) incorporation, respectively. All the experiment had a control for osmolarity, i.e. media containing iso-osmolar mannitol concentrations (NG + 24.5 mM mannitol).

Methods

Metabolic and cardiovascular parameters. Body weights were measured twice a week and served as a guide for supportive insulin treatment. Urine volume was measured at 3-month intervals. Blood glucose levels were measured biweekly by the use of an automated colorimetric instrument (Glucocard Memory 2®, Menarini Diagnostics, Florence, Italy) from blood obtained by tail venepuncture. Glycated Hb levels were assessed by boronate affinity gel chromatography using the Glyc-Affin GHb kit (PerkinElmer, Norwalk, CT) [23]. Blood pressure was recorded by the tail cuff method.

Renal function. Total proteinuria was measured using the Bradford dye-binding protein assay kit (Pierce Chemical, Rockford, IL), whereas serum creatinine was assessed by the Jaffe method [23].

Renal structure. Renal morphology was evaluated semi-quantitatively in sections stained with periodic acid Schiff (PAS) [23]. At least 60 glomerular tuft profiles per sample, selected on PAS stained sections by moving from external to deep cortex in a serpentine manner (with exclusion of profiles containing <3 mesangial tracts), were analyzed. A pathologist blinded to the group assignment of the specimens scored mesangial expansion; in each experimental animal, the mean score of each rat was derived from the individual scores of all the glomeruli. The expansion of mesangial matrix was scored (scale 0 to 4) as previously reported [26] and glomerular sclerosis was assessed as percent of involved glomeruli [23]; ~150 glomerular tuft profiles per animal were evaluated. Tubulo-interstitial damage was evaluated using a semi-quantitative scale (0: absent; 1: <10%; 2: 10–30%; 3: 30–70%; 4: >70%) and the extension of cortical lesions in each animal was expressed as the mean value obtained measuring a whole sagittal section [23].

ECM and TGF-β1 production. Transcripts for the ECM components fibronectin, laminin B1 and collagen IV z1 chain and the pro-sclerotic cytokine TGF-β1 were measured by competitive RT-PCR [23]. Total RNA was extracted from isolated glomeruli and mesangial cells by the guanidine thiocyanate–phenol–chloroform method using Trizol (Invitrogen Italia SRL, San Giuliano Milanese, Italy) and the purity of RNA preparation was confirmed by an absorbance 260/280 ratio >1.9, as measured in a Beckman DU-65 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). Then, 1 μg of total RNA was reverse transcribed using Retroscript kit (Ambion, Austin, TX). The following primers were used: fibronectin sense 5’-AGG GTG GTG TGC TAC TCT GT-3’—antisense 5’-GAT GCA CTG ATT CCT TCG TGA TG-3’—laminin B1 sense 5’-TTG CAG TCA CCT GCA GGA TG-3’—antisense 5’-CAG CCA GAC GAT AG-3’; collagen IV z1 chain sense 5’-TCT TTG CTA TGG TG-3’—antisense 5’-ATA CAG GCC TTT CGC TTC AG-3’; TGF-β1 sense 5’-ATG CAG GGC TTT CGC TTC AG-3’—antisense 5’-GTC CAG GCT CCA AAC AGT GA-3’; collagen I sense 5’-TCT CAG CGG TTC TCT GTA TGG GA-3’—antisense 5’-GAG CAC CAA GGT GTG-3’—antisense 5’-TCA TGA GGT AGT CCG TCA GG-3’. Competitive PCR was performed by using increasing amounts of mutants made by creating a deletion in the original PCR product and preliminary experiments were performed to establish the range of mutant concentrations producing a slope of the line close to one and within which the equivalence point falls. After electrophoresis of PCR products, the ratio of unknown cDNA/mutant was quantified by scanning densitometry using the ImageJ software, a public domain Java image processing program inspired by NIH Image, and results were expressed as the ratio of each target to β-actin mRNA level. The levels of fibronectin in conditioned media from cultured mesangial cells were quantified by ELISA using a rabbit
polyclonal antibody against rat fibronectin (Calbiochem), whereas release of total and bioactive TGF-β1 was measured using the Quantikine TGF-β1 Kit (R&D Systems), with and without prior acidification, respectively. Values were normalized to the DNA content of monolayers, as assessed fluorimetrically in 0.5 N NaOH extracts after reaction with 0.6 μM 4,6-diamidino-2-phenylindole (Sigma), as previously described [24].

**GLUT1 protein expression.** GLUT1 protein expression in kidney sections was measured by immunofluorescence, as previously reported [27]. Briefly, 5-μm-thick acetone-fixed kidney sections were sequentially hydrated and incubated for 1 h at room temperature with a rabbit polyclonal antibody raised against human GLUT1 and crossreacting with the rat and mouse protein (Diagnostic International, Schriesheim, Germany). Then, sections were incubated for 30 min at room temperature with a fluorescein isothiocyanate-labelled goat anti-rabbit secondary antibody (Alezafluor 488, Molecular Probe). Specificity of antibody labelling was demonstrated after substituting proper control immunoglobulins (Zymed, Histo-Line Laboratories, Milan, Italy) for the primary antibody. Slides were mounted with Vectashield aqueous mounting medium (Vector Laboratories, DBA Italia SRL, Milan, Italy) and observed and photographed using a Nikon Eclipse E600 microscope (Nikon Europe B.V., Badhoevedorp, The Netherlands). A semi-quantitative evaluation (0 = negative, 1 = mild positivity, 2 = intense positivity) of GLUT1 staining was performed by examining 30 glomeruli per section. GLUT1 protein expression in mesangial cell cultures was measured by western blot analysis.

Cells were grown in 100 mm Petri dishes (Falcon) under the above experimental conditions, then total or plasma membrane extracts were obtained. To obtain total cell lysates [28], cells were scraped, pelleted by centrifugation, disrupted by incubation for 15 min at 4 °C in lysing buffer (25 mmol/l Tris-HCl, pH 7.4, 0.2% sodium dodecyl sulfate (SDS), 50 mmol/l NaCl, 0.5% sodium deoxycholate, 2% Nonidet P-40 (NP-40), 1 mmol/l phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml aprotinin, 2 μg/ml leupeptin) under continuous agitation, and centrifuged to collect the supernatant. To obtain plasma membrane extracts [29], cells were scraped and homogenized in a ground glass dounce homogenizer at 4 °C using an ice-cold buffer consisting of PBS containing 200 mmol/l HEPES, 1 mmol/l disodium ethylenediaminetetraacetic acid (Na₂EDTA), 30 mmol/l KCl, 100 mmol/l MgCl₂, 2 mmol/l PMSF, 10 mmol/l benzamidine, 2 mmol/l dithiotreitol (DTT), 25 μg/ml leupeptin and 6 μg/ml aprotinin, then centrifuged at 500 g for 15 min at 4 °C to pellet unbroken cells and nuclei. The supernatant was centrifuged at 40 000 g for 2 h at 4 °C to collect the membrane fraction, which was washed three times in the extraction buffer and resuspended in a lysis buffer containing 0.1% SDS and NP-40 Triton-X-100 protease inhibitors. Extracts were assayed for protein content by the Bradford dye-binding protein assay kit (Pierce Chemical). For western blot analysis [28,29], protein samples (10–15 μg) were added with an equal volume of sample buffer 2x (100 mmol/l Tris-HCl, pH 7.4, 5% SDS, 10% saccharose, 1 mmol/l Na₃EDTA, 0.025% bromophenol blue, 0.1 mol/l DTT), separated by SDS-polyacrylamide gel electrophoresis (PAGE; 10% acrylamide, Bio-Rad Laboratories, Hercules, CA) and transferred by electroblotting using a MINI PROTEAN II™ (Bio-Rad Laboratories) onto polyvinylidene difluoride membranes (Amersham, Amersham, UK). The membranes were incubated overnight at 4 °C under agitation with Tris-buffered saline (TBS)-Tween (TBS + 0.5% Tween 20) +5% nonfat dry milk (NFDM) to block the nonspecific reactivity, then probed for 1 h at room temperature under agitation with the rabbit polyclonal anti-GLUT1 antibody, diluted 1:100 in TBS-Tween +3% NFDM. Subsequently, the membranes were incubated for 45 min at room temperature with a goat anti-rabbit IgG antibody conjugated with peroxidase (Dako), diluted 1:1500 in TBS-Tween +3% NFDM, washed and developed with ECL reagent (Amersham). Immunocomplexes were revealed by autoradiography and quantified by scanning densitometry using the ImageJ software. Results of total and cytosolic fraction analysis were normalized to the signal of β-actin, revealed by the use of a goat polyclonal antibody raised against the C-terminus of human β-actin (Santa Cruz Biotechnology, Santa Cruz, CA), diluted 1:1000.

**Glucose transport.** Confluent monolayers grown in 16 mm multi-well culture dishes (Falcon, Becton Dickinson, Lincoln Park, NJ) under the above experimental conditions. [³H]DOG incorporation was assessed after 15 min incubation at 37°C in 95% air and 5% CO₂ humidified atmosphere under stirring conditions [2,11]. For this purpose, 1 μCi of [³H]DOG (12 Ci/mmol, Amersham) in PBS with Ca and Mg was added to each well and, at the end of the incubation period, the incorporation was stopped by addition of 1 ml ice-cold PBS containing 20 mmol/l glucose. Monolayers were then washed three times with PBS, solubilized overnight at 4°C in 1 ml 0.1% SDS and processed for liquid scintillation counting in a Tri-Carb 2100 TR liquid scintillation analyzer (Packard Instruments, Meriden, CT). Results of [³H]DOG uptake were normalized per protein content of each well, as measured by the Bradford method on 10 μl aliquots of samples using BSA as standard. To measure glucose transport kinetics, [³H]DOG incorporation was measured over 5 min in the presence or absence of increasing amounts (0.1–5 mM) of unlabelled 2-deoxyglucose (Sigma) [11]. Data were analysed by Hanes plot analysis and maximal velocity (Vmax) and Michaelis-Menten constant (Km) were calculated.

**Statistical analysis**

Values are expressed as mean ± SD; the percent change was also calculated. Statistical significance was evaluated by one-way ANOVA followed by the Student–Newman–Keuls test for multiple comparisons. All statistical tests were performed on raw data.

**Results**

**In vivo studies**

**Metabolic and cardiovascular parameters.** Impairment of body growth as well as increases in blood glucose and glycated Hb levels and urine volume induced by diabetes were similar in the two rat strains and attested to the presence of severe metabolic derangement (Table 1). As previously reported [23], blood pressure...
was normal in the MNS and elevated in the MHS throughout the study, and was unaffected by diabetes (data not shown).

Renal function. As previously shown [22], both proteinuria and serum creatinine were similar in the two rat strains at time 0. Proteinuria increases progressively with time in MNS rats (>30-fold increment at 6 months vs time 0, P < 0.001), with significantly higher levels in diabetic vs nondiabetic animals at 3, but not 6 months. Conversely, proteinuria did not change with either age or diabetes in the MHS rats. Serum creatinine also increased with age in MNS rats (P < 0.001), with significantly higher values in both nondiabetic and diabetic MNS at 6, but not 3 months, as compared with the corresponding MHS rats (Table 2).

Renal structure. As previously described [17,22], no sign of glomerular disease was detected in the two strains at time 0 and in MHS rats at both 3 and 6 months, whereas MNS rats showed clear evidence of glomerular disease, particularly at 6 months. In keeping with our previous morphometric data [23], mesangial expansion, but not glomerular sclerosis and tubulo-interstitial damage, was more pronounced (+87%) in diabetic vs nondiabetic MNS rats; conversely, no change in these parameters was detected in diabetic vs nondiabetic MHS rats (Table 2).

ECM and TGF-β1 gene expression. Transcripts for the ECM components fibronectin, laminin B1 and collagen IV α1 chain and the prosclerotic cytokine TGF-β1 were similar in the two rat strains at time 0 (data not shown). The gene expression for these proteins increased significantly with age in the MNS (by >30%, P > 0.001, except for laminin), but not in the MHS rats. At 3 months of disease duration, higher mRNA levels (fibronectin +38%, laminin B1 +39%, collagen IV α1 chain +27%, and TGF-β1 +29%, P < 0.001) were detected in the diabetic vs nondiabetic MNS rats; at 6 months, ECM and TGF-β1 gene expression was still increased in the diabetic MNS vs the corresponding nondiabetic controls, with maximal increase observed for collagen IV (+49%) (Figure 1). No significant increase was detected in the diabetic vs nondiabetic MHS rats, at both 3 months (data not shown) and 6 months (Figure 1).

GLUT1 expression. At time 0, GLUT1 protein was detected in glomeruli from nondiabetic MNS rats, though at a low level of positivity, but not in those from MHS rats; GLUT1 expression increased significantly in nondiabetic MNS (from 0.14 ± 0.03 to 0.34 ± 0.12, P < 0.01), but not MHS rats throughout the study. GLUT1 fluorescence was increased in diabetic vs nondiabetic MNS rats at 3 months (0.56 ± 0.20 vs 0.20 ± 0.05, P < 0.01) and 6 months (1.01 ± 0.18 vs 0.34 ± 0.12, P < 0.001) of disease duration, whereas it was only barely detectable in MHS rats, in which it was localized mainly in podocytes (Figure 2).
Table 2. Effect of diabetes and aging on renal function, as assessed as proteinuria (mg/24 h) and serum creatinine (μmol/l), and structure, as assessed as kidney wet weight (g), mesangial expansion (score), glomerular sclerosis (%), and tubulo-interstitial lesions (score), in time 0 (0) MNS and MHS rats and diabetic (D3 and D6) and age-matched nondiabetic (ND3 and ND6) MNS and MHS rats at 3 and 6 months of disease duration (mean±SD; n = 6 animals in per group, except for proteinuria at time 0, n = 18, and 3 months, n = 12).

<table>
<thead>
<tr>
<th>MNS (time)</th>
<th>MHS (time)</th>
<th>MNS (time)</th>
<th>MHS (time)</th>
<th>MNS (time)</th>
<th>MHS (time)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nd3</td>
<td>D3</td>
<td></td>
<td>Nd3</td>
<td>D3</td>
</tr>
<tr>
<td>Proteinuria</td>
<td>20.76±5.34</td>
<td>22.03±6.73</td>
<td>266.05±160.96</td>
<td>464.08±206.23a</td>
<td>22.43±3.80a</td>
</tr>
<tr>
<td>Serum creatinine</td>
<td>28.34±1.28</td>
<td>28.73±2.16</td>
<td>29.74±2.12</td>
<td>30.29±1.68</td>
<td>29.62±1.20</td>
</tr>
<tr>
<td>Kidney wet weight</td>
<td>2.30±0.18</td>
<td>2.20±0.11</td>
<td>2.73±0.20</td>
<td>3.09±0.30</td>
<td>3.12±0.26</td>
</tr>
<tr>
<td>Mesangial expansion</td>
<td>0.12±0.07</td>
<td>0.11±0.05</td>
<td>0.58±0.15</td>
<td>1.25±0.37a</td>
<td>0.19±0.08b</td>
</tr>
<tr>
<td>Glomerular sclerosis</td>
<td>0.02±0.01</td>
<td>0.01±0.01</td>
<td>10.46±3.47</td>
<td>10.19±2.06</td>
<td>0.29±0.15b</td>
</tr>
<tr>
<td>Tubulo-interstitial lesions</td>
<td>0.02±0.01</td>
<td>0.01±0.01</td>
<td>0.72±0.30</td>
<td>0.74±0.27</td>
<td>1.97±0.73</td>
</tr>
</tbody>
</table>

Significantly different at *P<0.001 or †P<0.01 vs the corresponding nondiabetic rats and ‡P<0.001 or §P<0.01 vs the corresponding MNS rats.
HG vs NG conditions, mesangial cells from 1-month-old animals showed enhanced \[^{3}H\]DOG uptake with increases which were significantly more marked in those from MNS vs MHS rats (\(+47\%\) vs \(+27\%, P < 0.001\)). Iso-osmolar mannitol did not affect \[^{3}H\]DOG uptake by monolayers. Co-incubation with TGF-\(\beta\) produced a further stimulation of \[^{3}H\]DOG incorporation in both mesangial cell lines, with more pronounced increases vs HG-treated monolayers in MNS than in MHS mesangial cells (\(+74\%\) vs \(+53\%)\) (Figure 5A).

Fig. 1. Glomerular mRNA levels of fibronectin (A), laminin B1 (B), collagen IV \(\alpha 1\) chain (C) and TGF-\(\beta1\) (D) (expressed as OD ratio to \(\beta\)-actin mRNA level) from diabetic (black bars) and age-matched nondiabetic (white bars) MNS and MHS rats at 6 months of disease duration (mean \(\pm\) SD; \(n = 4\) per group). Significantly different at *\(P < 0.001\) vs the corresponding nondiabetic rats and or †\(P < 0.001\) vs the corresponding MNS rats.

Fig. 2. Glomerular protein expression of GLUT1 in MNS and MHS rats. Representative kidney sections from rats of the MNS (nondiabetic, A and diabetic, C) and the MHS (nondiabetic, B and diabetic, D) at 6 months of disease duration (100× magnification).
HG-induced increases were prevented by co-incubation with α-TGF-β blocking antibody, but not control chicken IgG (data not shown). Kinetic analysis showed that Vmax, but not Km (data not shown), increased in response to HG and/or TGF-β in cells obtained from both strains, again to a more pronounced extent in MNS than in MHS cells (Figure 5C). Again, similar results were obtained in cells from 8-month-old animals (Figure 5B–D).

Discussion

In the in vivo studies, diabetic MNS rats developed DN superimposed onto spontaneous glomerulosclerosis, whereas diabetic MHS rats remained free of renal disease, as reported in our previous publication [23]. Glomerular expressions of GLUT1 protein and ECM and TGF-β mRNA were significantly upregulated in diabetic vs non-diabetic MNS, but not MHS rats, as compared with the corresponding age-matched non-diabetic controls. In the in vitro studies, mesangial cells isolated from 1- and 8-month-old MNS rats showed higher glucose transport activity and GLUT1 membrane expression than those from age-matched MHS rats, when exposed to and/or TGF-β levels. Likewise, ECM (but not TGF-β) production increased more markedly in response to HG and/or TGF-β in MNS vs MHS mesangial cells, as previously reported [24].

In our previous publication [23], MHS rats were found to be protected from diabetes-induced haemodynamic changes, at variance with MNS, which showed increased GFR and filtration fraction, indicating an increase of efferent/afferent resistance with consequent

---

**Table 3.** Medium fibronectin (μg/μg DNA) and total TGF-β1 (ng/μg DNA) release from mesangial cells isolated from 1- and 8-month-old MNS and MHS rats and incubated under HG and NG conditions for 10 days (mean±SD; n=4 per group in 3–4 replicates per experimental condition).

<table>
<thead>
<tr>
<th></th>
<th>Fibronectin</th>
<th>Total TGF-β1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NG</td>
<td>HG</td>
</tr>
<tr>
<td>1 month</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MNS</td>
<td>0.950±0.039</td>
<td>1.766±0.083</td>
</tr>
<tr>
<td>MHS</td>
<td>0.921±0.055</td>
<td>1.435±0.076</td>
</tr>
<tr>
<td>8 months</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MNS</td>
<td>1.155±0.064</td>
<td>2.335±0.242</td>
</tr>
<tr>
<td>MHS</td>
<td>0.964±0.050</td>
<td>1.492±0.065</td>
</tr>
</tbody>
</table>

Significantly different at *P<0.001 vs the corresponding NG monolayers, †P<0.001 or ‡P<0.01 vs the corresponding cells from MNS rats, and §P<0.001 or ‖P<0.01 vs cells from the corresponding 1-month-old rats.
increment of glomerular capillary pressure, driving enhanced GFR. In the present study, virtually no upregulation of the TGF-β/GLUT1 axis (and no ECM overproduction and renal disease) occurred in the haemodynamically-protected MHS rats, at variance with the glomerulosclerosis-prone MNS rats, in which glomerular GLUT1 expression increased progressively, in parallel with ECM and TGF-β gene expression and glomerular injury. Upregulation of the TGF-β/GLUT1 axis under diabetic conditions did not lead to a further increment of glomerular sclerosis nor to differences in the appearance of sclerotic lesions, but only to a more marked matrix deposition than nondiabetic animals, as reported in our previous publication [23]. Conversely, HG was capable of inducing changes in TGF-β/GLUT1 axis and ECM production in mesangial cells isolated from MHS rats, i.e. under...
conditions in which haemodynamics is not operating, though to a significantly lesser extent than in mesangial cells isolated from MNS rats (except for TGF-β production).

These results confirm and extend previous observations from Gnudi et al. in the hypertensive renal disease occurring in the Dahl-S rat, but not in the young SHR, showing that glomerular hypertension and injury correlate to the activation of the TGF-β/GLUT1 axis [14]. Our data indicate that the TGF-β/GLUT1 axis plays a major role also in the pathogenesis of DN by linking the haemodynamic and metabolic abnormalities triggered by hyperglycaemia. Hence, the permissive role of haemodynamic changes toward hyperglycaemia-induced injury would be mediated by the upregulation of this axis induced by stretching (possibly amplified by hyperglycaemia), but not by hyperglycaemia alone. The view that this glucose transporter is induced predominantly by haemodynamic stimuli is further supported by a recent report in mice carrying the oligosyndactyly (Os) allele, which have a 50% reduction in nephron number and are susceptible to (haemodynamically-mediated) renal disease. ragged oligosyndactyly pintail (ROP) Os/+ mice backcrossed into the mouse strain (fvb) background developed rapidly progressive renal disease and renal failure at variance with classical ROP Os/+ mice, showing only glomerulosclerosis, and also had higher glomerular GLUT1 expression and glucose uptake [30].

The finding that upregulation of GLUT1-dependent glucose transport and ECM production in response to TGF-β, either stimulated by HG or added to the culture medium, was more marked in mesangial cells from MNS than MHS rats suggests an increased activity of the TGF-β/GLUT1 axis in the glomerulosclerosis-prone MNS rats. This conclusion is supported by the observation that HG-induced changes in glucose transport, GLUT1 and ECM in the MNS were more marked than those detected not only in mesangial cells from the glomerulosclerosis-resistant MHS rats, but also in cells isolated from Sprague–Dawley or other rat strains (unpublished observations). The observation that GLUT1 and ECM, but not TGF-β expression, was more pronounced in MNS vs MHS mesangial cells suggests that, in the MNS, a similar HG-induced TGF-β upregulation resulted in an increased response vs the MHS in terms of GLUT1 and consequent glucose transport activity, which produced a more marked ECM deposition, independent of further, glucose-dependent TGF-β induction. This view is in keeping with a recent report indicating that increased GLUT1 expression, independent of elevated extracellular glucose levels, can directly enhance ECM production through a PKC and AP-1 dependent pathway, without causing mitogen-activated protein kinase activation, TGF-β induction or reactive oxygen species generation [6], which have all been implicated in the pathogenesis of DN.

This increased activity of the TGF-β/GLUT1 axis in the MNS rats seems to be genetically determined, since an enhanced GLUT1 response to TGF-β upregulation was detectable in mesangial cells isolated from them before the onset of spontaneous renal disease. It underlines the susceptibility to DN in MNS rats, by amplifying the effects of hyperglycaemia and associated TGF-β upregulation on ECM overproduction, thus leading to excess matrix deposition under diabetic conditions.

In conclusion, these results indicate that the TGF-β/GLUT1 axis plays a major role also in the pathogenesis of DN by linking the haemodynamic and metabolic abnormalities triggered by hyperglycaemia, with the permissive role of haemodynamic changes toward hyperglycaemia-induced injury being mediated through the upregulation of this axis. These data also indicate that susceptibility to diabetic glomerulopathy in MNS rats is associated with increased (genetically-determined) GLUT1-dependent glucose transport activity in response to hyperglycaemia and/or TGF-β, which may amplify ECM overproduction occurring under diabetic conditions.

Acknowledgements. This work was supported by grants from the Ministry of Education of Italy (40 and 60%), the Ministry of Health of Italy, the International Center for the Study of Diabetes, and the Diabetes, Endocrinology and Metabolism Foundation, Rome, Italy.

We are indebted to Prof. Giuseppe Bianchi (Division of Nephrology and Hypertension, San Raffaele Hospital, ‘Vita e Salute’ University, Milan, Italy) for providing us with the Milan rats.

Conflict of interest statement. None declared.

References


Received for publication: 3.10.05
Accepted in revised form: 3.1.06