High glucose induces macrophage inflammatory protein-3α in renal proximal tubule cells via a transforming growth factor-β1 dependent mechanism

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Abstract

Background. Hyperglycaemia is a causative factor in the pathogenesis of diabetic nephropathy, known to induce chemokines in the kidney. Macrophage inflammatory protein-3α (MIP-3α) is a CC chemokine that has been reported to attract memory T lymphocytes. Our previous microarray study showed significant increased level of MIP-3α in high glucose-induced transcriptional profile in renal proximal tubule cells. Transforming growth factor-β1 (TGF-β1) is a key regulator in inflammation and fibrosis in diabetes mellitus setting.

Methods. This study aimed to determine the role of TGF-β1 in high glucose-induced MIP-3α expression. An in vitro model of human proximal tubular cells (HK-2 cells) and an in vivo model of the transgenic (mRen-2)27 diabetic rat, well characterized as a model of human diabetic nephropathy, were used. Small interfering RNA technology was used to silence TGF-β1 gene in HK-2 cells and subsequent experiments were performed to measure mRNA and protein levels of MIP-3α using real time reverse transcription–polymerase chain reaction (RT–PCR) and enzyme-linked immunosorbent assay (ELISA). Immunohistochemistry was used to measure the protein level of MIP-3α and CD3 a marker of T lymphocytes in the in vivo model.

Results. MIP-3α mRNA and protein expression was increased in HK-2 cells by high glucose and TGF-β1. MIP-3α was up-regulated in the dilated tubules of diabetic rats compared with non-diabetic control animals and CD3 was found to be present around the dilated tubules expressing MIP-3α. This up-regulation was attenuated in the presence of an angiotensin-converting enzyme (ACE) inhibitor. MIP-3α expression significantly decreased in cells in which the TGF-β1 gene was silenced using small interfering RNA. Furthermore, exposure to high glucose did not induce MIP-3α expression in TGF-β1 gene silenced cells compared with wild-type cells.

Conclusions. In summary, we have uniquely demonstrated that high glucose increases MIP-3α through a TGFβ1 dependent pathway, suggesting the centrality of TGF-β1 in both the inflammatory and previously demonstrated fibrotic responses in diabetic nephropathy.

Keywords: high glucose; macrophage inflammatory protein-3α (MIP-3α); renal tubular cells; transforming growth factor-β1 (TGF-β1)

Introduction

Hyperglycaemia is recognized to be the key factor driving renal functional and pathological changes in diabetic nephropathy. Many lines of evidence from our own and other laboratories suggest that high glucose induces inflammation in the human kidney and chemokines play an important role in inflammatory kidney diseases [1–3]. Macrophage inflammatory protein-3α (MIP-3α), also known as chemokine (C-C motif) ligand 20 (CCL20), liver and activation-regulated chemokine (LARC) and Exodus-1, is a CC chemokine that has been reported to attract memory T lymphocytes. Chemokine receptor CCR6 is an exclusive receptor for MIP-3α and the ligand-receptor (MIP-3α-CCR6) has been reported to chemoattract immature dendritic cells (DC) [4,5]. MIP-3α was first identified and cloned in 1997. The human MIP-3α gene
was mapped to chromosome 2q33–37 and its promoter region contains possible binding sites for nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) which are known to be involved in the transcriptional regulation of various inflammatory cytokines and chemokines [4]. Both MIP-3α and CCR6 have been reported to be markedly up-regulated in chronic inflammatory skin disorders [4].

Transforming growth factor-β1 (TGF-β1) is a well-recognized inflammatory cytokine in the kidney playing a central role in progressive kidney diseases including diabetic nephropathy [6–8]. We have recently demonstrated TGF-β1 induced interleukin-8 (IL-8) and macrophage chemoattractant protein-1 (MCP-1) in a human proximal tubule cell line [8]. Sato et al. [9] demonstrated that TGF-β1 induced CCR6 protein expression in monocytes. However, the role of TGF-β1 in regulating MIP-3α expression in the kidney is unknown.

The aim of this work was to determine the effect of high glucose on MIP-3α and the dependence of MIP-3α expression on TGF-β1 in an in vitro model of proximal tubule cell line (HK-2 cells). The observations were confirmed in the diabetic transgenic Ren-2 rat.

**Research designs and methods**

**Cell culture**

HK-2 cells, a proximal tubule cell line from American Type Cell Collection (ATCC, USA), were used in this study.

To determine the effect of high glucose on MIP-3α, HK-2 cells were exposed to 5 and 30 mM D-glucose for 72 h. RNA and supernatant was collected to determine the level of MIP-3α mRNA and protein expression. HK-2 cells were then exposed to high glucose for 1, 3, 5 and 10 days and conditioned media was collected and centrifuged at 3000 rpm and 4°C for 10 min to remove cell debris and then stored at -80°C for MIP-3α enzyme-linked immunosorbent assay (ELISA) (R&D systems, Minneapolis, MN, USA).

To determine the effect of TGF-β1 on MIP-3α expression, HK-2 cells were exposed to 2ng/ml TGF-β1 for 72 h. RNA and conditioned media were collected for measurement of MIP-3α mRNA and protein levels.

To determine the role of TGF-β1 in high glucose-induced MIP-3α expression, 30 nM of TGF-β1 siRNA (Ambion, USA) was introduced into HK-2 cells using Lipofectamine 2000 (Invitrogen, USA) according to manufacturer's instructions. In parallel, cells were transfected with 30 nM non-specific siRNA and transfection reagent only (designed as Mock) which served as the control data sets. Cells were then treated with or without 30 mM D-glucose for 72 h. Both RNA and conditioned media were collected for verification of TGF-β1 gene silenced as described previously [10] and measurement of MIP-3α mRNA and protein expression.

**Real-time reverse transcription polymerase chain reaction (real-time RT–PCR)**

Both water blank and non-reverse transcribed RNA samples were used as negative controls. Briefly, total RNA (2 μg) was treated with DNase I (Invitrogen, USA) and then cDNA was synthesized using reverse transcriptase Superscript II RT (Invitrogen, USA). Sequence-specific primers for human MIP-3α (accession no. NM_004591) and β-actin (accession no. NM_001101) were as follows: MIP-3α: Forward 5’-AGAGTTTGCTCTCTGGCTG-3’; Reverse: 5’-GGATGAAGAATTACGGTCTGTG-3’; and β-actin: Forward 5’-GCTCGTCGTGCACGACCGC-3’; Reverse 5’-CAACATGATCTGCTTCATTCTC-3’. Primers specificity in real-time PCR reactions was confirmed using RT–PCR. A 25 μl of real-time PCR reaction included Brilliant SYBR Green QRT–PCR Master Mix according to manufacturer’s instructions (Stratagene, USA). Real-time quantitations were performed on the Bio-Rad iCycler iQ system (CA, USA). The fluorescence threshold value was calculated using the iCycler iQ system software. The calculation of relative change in mRNA was performed using the delta-delta method [11], with normalization for the housekeeping gene β-actin.

**In vivo studies in diabetic ren-2 rats**

Eight-week-old female, homozygous (mRen-2)27 rats (St. Vincent’s Hospital Animal House, Melbourne, Australia) weighing 170 ± 20 g were randomized to receive either 55 mg/kg of streptozotocin (STZ; Sigma, St Louis, MO, USA) diluted in 0.1 M citrate buffer pH 4.5 or citrate buffer (non-diabetic) by tail vein injection following an overnight fast [7,12]. Each week, rats were weighed and their blood glucose was determined using an AMES glucometer (Bayer Diagnostics, Melbourne, Australia) and only STZ-treated animals with blood glucose > 20 mmol/l were considered diabetic. Every 4 weeks, systolic blood pressure (SBP) was determined in pre-heated conscious rats via tail-cuff plethysmography using a non-invasive blood pressure (NIBP) controller and Powerlab (AD instruments, NSW, Australia). All animals were housed in a stable environment maintained at 22 ± 1°C with a 12 h light/dark cycle commencing at 6 am. Diabetic rats received twice a week injection of insulin (2–4 units intraperitoneally; Humulin NPH, Eli Lilly and Co., Indianapolis, IN) to reduce mortality and to promote weight gain. In the experimental group of diabetic rats treated with angiotensin-converting enzyme (ACE) inhibitor, rats were fed with perindopril (0.2 mg/kg/ day) via drinking water. Experimental procedures adhered to the guidelines of the National Health and Medical Research Council of Australia’s Code for the Care and Use of Animals for Scientific Purposes.
and was approved by the Animal Research Ethics Committee of St. Vincent's Hospital.

**Tissue preparation and immunohistochemistry**

Rats were anaesthetized (Nembutal 60 mg/kg body weight i.p. Boehringer–Ingelheim, Australia) at week 12 and the abdominal aorta cannulated with an 18G needle. Perfusion-exsanguination commenced at SBP (180–220 mmHg) via the abdominal aorta with 0.1 M PBS, pH 7.4 (20–50 ml) to remove circulating blood and the inferior vena cava adjacent to the renal vein was simultaneously severed allowing free flow of the perfusate. After clearance of circulating blood, 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 was perfused for a further 5 min (100–200 ml of fixative). Kidneys were then excised, de-capsulated, sliced transversely, immersed into 4% paraformaldehyde in 0.1 M phosphate buffer for overnight fixation and then paraffin-embedded for subsequent light microscopic evaluation [7].

**Immunohistochemistry for MIP-3α**

In brief, four micron sections were placed into histosol to remove the paraffin wax, re-hydrated in graded ethanol and immersed into tap (dH₂O) water before being incubated for 20 min with normal swine serum diluted 1:10 with 0.1 mol/l PBS, pH 7.4. Sections were then incubated with rabbit anti-MIP-3α (abcam, Cambridge, UK) diluted 1:200 and rabbit anti-CD3 (Dako, Denmark) diluted 1:50, respectively with PBS overnight (18 h) at 4°C. The following day the sections were thoroughly washed in PBS (3 × 5 min changes), incubated with 3% hydrogen peroxide for 10 min to block endogenous peroxidase, then rinsed with PBS (2 × 5 min) and incubated with biotinylated swine anti-rabbit IgG antibody (DAKO, Carpenteria, CA, USA), diluted 1:200 with PBS. Sections were rinsed with PBS (2 × 5 min) followed by incubation with an avidin-biotin peroxidase complex (Vector, Burlingame, CA, USA), diluted 1:200 with PBS. Following rinsing with PBS (2 × 5 min), localization of the peroxidase conjugates was achieved by using diaminobenzidine tetrahydrochloride as a chromagen, for 1–3 min. Sections were then incubated in tap water for 5 min to stop reaction and then counterstained in Mayer’s haemotoxylin, in Scott’s tap water, dehydrated, cleared and mounted in DPX. Sections incubated with 1:10 normal swine serum, instead of the primary antiserum, served as the negative controls [7].

**Statistical analysis**

Real time RT–PCR results were expressed as a fold change compared with the control value. MIP-3α ELISA data are expressed as absolute values (pg/ml). Each experiment was performed independently a minimum of three times. Results are expressed as mean ± SEM. Statistical comparisons between groups were made by analysis of variance (ANOVA), with pairwise multiple comparisons made by Fisher’s protected least-significant difference test. Analyses were performed using the software package, Statview version 4.5 (Abacus Concepts Inc., Berkley, CA, USA). P values < 0.05 were considered significant.

**Results**

**MIP-3α expression in HK-2 cells exposed to high glucose and in vivo model of diabetic Ren-2 rats**

Exposure of HK-2 cells to 30 mM D-glucose (high glucose) for 72 h significantly increased MIP-3α mRNA to 2.4 ± 0.12-fold (P < 0.005) compared with cells exposed to 5 mM D-glucose (normal glucose) (Figure 1A). Protein level of MIP-3α also increased by 1452 ± 100 pg/ml after 72 h high glucose exposure (P < 0.005) (Figure 1B). Osmotic control (30 mM L-glucose) did not change the expression levels of MIP-3α, suggesting that the high glucose induced MIP-3α was not due to osmotic stress on the cells, but specific to the high extracellular glucose concentrations. We have demonstrated by microarray analysis that high glucose up-regulated MIP-3α expression level when HK-2 cells were exposed to high glucose for 11 days (Qi et al. Am J Pathology, In press). High glucose significantly increased MIP-3α protein at 72 h which was sustained to day 10 (P < 0.0005; Figure 1C). Transgenic Ren-2 rats rendered diabetic with streptozotocin [7,12] were used as an in vivo model to assess the level of MIP-3α in a model of progressive diabetic nephropathy. The animal characteristics are shown in Table 1. MIP-3α was significantly up-regulated in the dilated tubules of diabetic Ren-2 rats compared with non-diabetic Ren-2 rats after 12 weeks observation (Figure 2C). CD3, a marker of T lymphocytes, was specifically increased around dilated tubules expressing MIP-3α (Figure 2D). ACE inhibitor attenuated the increased level of MIP-3α and CD3 in diabetic rats (Figure 2E and F).

Numerous reports have demonstrated that the level of intrarenal TGF-β1 increased in diabetic conditions or induced by high glucose [13–15] and we have previously confirmed that high glucose induces TGF-β1 in HK-2 cells [1]. We hypothesized that high glucose up-regulated MIP-3α through a TGF-β1 dependent pathway. Subsequent studies were undertaken to test this hypothesis.

**The effect of TGFβ1 on MIP-3α expression in HK-2 cells**

Exposure of HK-2 cells to 2 ng/ml TGFβ1 for 72 h increased MIP-3α mRNA expression (P < 0.0005) (Figure 3A). MIP-3α protein expression increased by 4019 ± 1093 pg/ml (P < 0.05) after HK-2 cells were exposed to TGF-β1 for 72 h (Figure 3B). We further hypothesized that the endogenous TGF-β1 induced
by high glucose up-regulated MIP-3α in HK-2 cells. To confirm this hypothesis, we used siRNA technology which we have previously used to successfully silence TGF-β1 gene in HK-2 cells [10].

High glucose induced MIP-3α expression through a TGF-β1 dependent pathway

TGF-β1 gene was effectively silenced in HK-2 cells using siRNA technology and TGF-β1 mRNA level decreased by 90% as previously described [10]. The basal mRNA and protein levels of MIP-3α decreased significantly (both \( P < 0.05 \)) in TGF-β1 silenced cells compared with wild-type cells transfected with non-specific siRNA (Figure 4A and B). Moreover, exposure of HK-2 cells to high glucose for 72 h did not up-regulate MIP-3α mRNA or protein expression in TGF-β1 silenced cells compared with wild-type cells exposed to high glucose (\( P < 0.0005 \) and \( P < 0.005 \) respectively) (Figure 4C and D). These data suggest that high glucose induced MIP-3α expression through a TGF-β1 dependent pathway.

Table 1. Animal characteristics

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>( n )</th>
<th>BW (g)</th>
<th>SBP (mmHg)</th>
<th>HbA1c (%)</th>
<th>AER (mg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>329 ± 14</td>
<td>149 ± 6</td>
<td>3.57 ± 0.03</td>
<td>1.06 ± 0.15</td>
</tr>
<tr>
<td>Diabetic</td>
<td>8</td>
<td>285 ± 9*</td>
<td>172 ± 9.5*</td>
<td>12.6 ± 0.35**</td>
<td>8.67 ± 0.8**</td>
</tr>
<tr>
<td>Diabetic+ACEi</td>
<td>8</td>
<td>302 ± 5</td>
<td>122 ± 5</td>
<td>10.23 ± 0.27**</td>
<td>1.62 ± 1.32</td>
</tr>
</tbody>
</table>

* \( P < 0.05 \) vs control. ** \( P < 0.01 \) vs control.
Fig. 2. MIP-3α and CD3 expression in diabetic Ren-2 rats. Representative photomicrographs of MIP-3α and CD3 immunohistochemistry in non-diabetic, diabetic Ren-2 rats and diabetic Ren-2 rats treated with ACE inhibitor (0.2 mg/kg/day) at a week 12 study. Magnification 350x.

Fig. 3. TGF-β1 up-regulated MIP-3α expression in HK-2 cells. HK-2 cells were exposed to 2 ng/ml TGF-β1 for 72 h. RNA and supernatant was collected for measurement of MIP-3α mRNA and protein expression by real time RT-PCR (A) and ELISA (B). Results are expressed as mean ± SEM. ***P < 0.0005 vs control; *P < 0.05 vs control. All experimental conditions were induplicated and cell culture preparation was repeated three times, giving six individual measurements in each experimental group.
or non-specific siRNA of MIP-3HK-2 cells. Cells were then treated with 30 mM D-glucose (high glucose) for 72 h. RNA and supernatant was collected for measurement. All experimental conditions were induplicated and cell culture preparation was repeated three times, giving six individual measurements.

Discussion

This study has uniquely demonstrated high glucose increased MIP-3α expression through a TGF-β1 dependent pathway in an in vitro model of human proximal tubular cell line. Moreover, we have confirmed an increased level of MIP-3α in the well characterized diabetic Ren-2 rat model.

MIP-3α is a chemokine that is mainly expressed in inflamed epithelium. MIP-3α has been reported to be expressed in epithelial cells, endothelial cells and fibroblasts in many organs [16–18]. Woltman et al. [16] reported that renal proximal tubular epithelial cells are active players in the attraction of leucocytes during renal inflammatory responses. Diabetic nephropathy has not traditionally been considered as primarily an inflammatory disease. However, infiltrating monocytes, macrophages, mast cells and T-cells have all been demonstrated in predominantly in the interstitium of diabetic renal disease. These cells are densely packed with vasoactive peptides and growth factors and are thought to locally contribute to tissue injury. Despite the increasing recognition that inflammation plays a key pathogenic role in the progression of diabetic nephropathy, the factors that mediate the inflammatory response are only just being defined. We and others have recently demonstrated that TGF-β1 up-regulates the inflammatory chemokines IL-8 and macrophage chemotactic protein-1 (MCP-1) [2,10] in the proximal tubule. However, recognition that MIP-3α is a major chemokine expressed by epithelial cells attracting immature dendritic cells [16] necessitated further investigation as to its role in diabetic nephropathy. The present study demonstrates it to be specifically up-regulated in both in vitro in proximal tubular cells in response to high glucose and TGF-β1. Furthermore, high glucose induced MIP-3α via a TGF-β1 dependent pathway. Hepatocyte growth factor (HGF) and other chemokines (IL-1β), TNF-α, IL-17) were previously reported to induce MIP-3α expression [19,20]. MIP-3α promoter contains the binding sites of transcription factor AP-1 and NF-κB [21] and induction of MIP-3α by TNF-α via NF-κB dependent activation.

In our in vivo model of diabetic nephropathy, CD3 which is a marker of T lymphocytes was found to be present around the dilated tubules expressing MIP-3α, suggesting that MIP-3α is chemoattractant for...
T lymphocytes [22]. We have previously demonstrated that the ACE inhibitor perindopril decreases TGF-β1 in diabetic Ren-2 rats [23]. Moreover, ACE inhibitor attenuated the increased level of MIP-3x in the current study, suggesting that up-regulation of MIP-3x in diabetic rats might be mediated via an angiotensin-TGF-β1 axis. We acknowledge that high blood pressure in diabetic Ren-2 rats might contribute to the increased level of MIP-3x. Tucci and colleagues [24] reported that overexpression of MCP-1 is predominant in hypertensive patients. Therefore, angiotensin and hypertension could synergistically contribute to the increased level of MIP-3x.

In summary, these studies have importantly demonstrated MIP-3x was up-regulated in the kidney proximal tubule by high glucose. The up-regulation of MIP-3x was shown to be clearly dependent on TGF-β1 pathway in high glucose conditions in a proximal tubule cell line model. Current therapies used in diabetic nephropathy have not specifically targeted inflammatory pathways; hence the current experiments suggest this may be a fruitful area to further explore.

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Conflict of interest statement. None declared.

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