Secretion of collagen type IV by human renal fibroblasts is increased by high glucose via a TGF-β-independent pathway

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Abstract

Background. Tubulointerstitial fibrosis is an important component of diabetic nephropathy, which is characterized by increased expression of interstitial extracellular matrix components and aberrant expression of the basement membrane component collagen type IV. The present study examined the effect of high ambient glucose and transforming growth factor-β1 (TGF-β1) on collagen secretion by human renal fibroblasts and proximal tubular epithelial cells (PTECs).

Methods. Human renal fibroblasts (TK173) and PTECs (HK2) were used to examine the effects of high glucose (25 mM d-glucose) and TGF-β1 (1 ng/ml) on collagen type I, III and IV secretion compared with control medium (5.5 mM glucose). Matrix components were measured by enzyme-linked immunosorbent assay (ELISA) and reverse transcription–polymerase chain reaction (RT–PCR).

Results. Renal fibroblasts are the main producers of the interstitial components collagen type I and type III, while collagen type IV was secreted predominantly by PTECs. However, renal fibroblasts were also able to secrete collagen type IV. Secretion of collagen type IV by fibroblasts was increased upon stimulation with TGF-β1, reaching levels comparable with those secreted by TGF-β1-induced PTECs. Moreover, high glucose stimulated increased collagen type IV secretion. Importantly, this could not be attenuated by neutralizing pan-specific anti-TGF-β antibodies.

Conclusions. These data show that renal fibroblasts secrete collagen type IV, which can be increased by high glucose independent of endogenous TGF-β. This suggests that as well as the increased expression of interstitial components, renal fibroblasts can contribute to the increased expression of the basement membrane component collagen type IV in tubulointerstitial fibrosis observed during diabetic nephropathy.

Keywords: collagen type IV; diabetic nephropathy; fibroblasts; proximal tubular epithelial cells; TGF-β; tubulointerstitial fibrosis

Introduction

Tubulointerstitial fibrosis is an important component of diabetic nephropathy. The degree of tubulointerstitial damage is closely correlated with renal function. It is characterized by thickening of the tubular basement membranes, tubular atrophy, infiltration of mononuclear cells, and increased deposition of extracellular matrix (ECM) components [1,2].

The cellular compartment of the tubulointerstitium is composed of tubular epithelial cells (TECs) and interstitial fibroblasts. TECs are the predominant cell type of the interstitium, and serve as a major source of pro-inflammatory cytokines, chemokines and profibrotic growth factors during tubulointerstitial fibrosis. Furthermore, TECs contribute to the excessive ECM deposition in the kidney [3]. The resident interstitial fibroblasts are major producers of ECM components. Although normally present in low numbers, fibroblasts are increased during fibrosis [4], which may play a role in the progression of fibrosis by excessive ECM deposition [5,6]. Furthermore, resident cells also undergo morphological and functional changes [7].

In normal kidneys, the production of different collagen molecules is compartmentalized, with TECs as the principal cells of collagen type IV production [8], and fibroblasts producing collagens type I and III [9,10]. In tubulointerstitial fibrosis, matrix components are abundantly upregulated [11,12]. In addition to quantitative changes, there is aberrant expression of novel matrix components. For instance, collagen type IV, normally expressed in glomerular and tubular...
basement membranes [13], is no longer restricted to the basement membranes, but is also expressed in the interstitium [8,11,12,14,15]. Production of this collagen type IV has been attributed predominantly to TECs [8]. Most studies with renal fibroblasts examined total collagen secretion [16]. There are only a few studies which have examined the expression of collagen type IV in renal fibroblasts [17]. There is still little known about its regulation and the relative contribution of renal fibroblasts and TECs to the aberrant expression of collagen type IV during fibrosis.

Growth factors play an important role in the regulation of ECM production. In particular transforming growth factor-β (TGF-β) has been demonstrated to be an important mediator of the fibrotic process [18]. TGF-β expression is increased in the diabetic kidney, and treatment of streptozotocin-induced diabetic mice with neutralizing anti-TGF-β antibodies attenuated kidney hypertrophy and ECM gene expression [19]. Although TGF-β plays a central role in diabetic nephropathy, TGF-β-independent processes are also thought to play a role. For instance, we have shown recently that the glucose-induced expression of interstitial components fibronectin and collagen type III was TGF-β independent in human renal fibroblasts [10].

In this study, we examined the effect of high ambient glucose and TGF-β on the secretion of the interstitial collagens type I and III, and the basement membrane component collagen type IV by human renal fibroblasts and proximal tubular epithelial cells (PTECs). We found that renal fibroblasts secrete collagen type IV, which was increased when fibroblasts were stimulated with TGF-β1 or high glucose, reaching levels comparable with those secreted by TGF-β1-induced PTECs. Furthermore, glucose-induced collagen type IV was independent of endogenous TGF-β. These results suggest that renal fibroblasts can contribute to the observed aberrant expression of collagen type IV in the tubulointerstitium during fibrosis induced by the hyperglycaemic environment via a TGF-β-independent pathway.

Materials and methods

Cell culture

SV40-transformed human renal fibroblasts TK173 (obtained from a normal kidney) and TK188 (obtained from a fibrotic kidney), and HK2 (immortalized human PTECs) have been characterized previously [10]. Dermal fibroblasts were grown from normal skin tissues, and cells were used between passages 2 and 5 of culture. Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM), in 75 cm² culture flasks inactivated fetal calf serum (contained 5.5 mM glucose, supplemented with 10% heat-

Dulbecco’s modified Eagle’s medium (DMEM), which yielded a product of 433 bp) and the α1 subunit of collagen

and grown in DMEM containing 10% FCS for 24 h to reach confluence. Subsequently, cells were brought to quiescence by placing them in DMEM with 0.5% FCS. After 24 h, cells were cultured for 4 days in 0.3 ml of medium alone, i.e. DMEM containing 5.5 mM d-glucose supplemented with 0.5% FCS, or medium containing the following additives: 25 mM d-glucose (Merck, Darmstadt, Germany), 25 mM l-glucose (Sigma) and 1 ng/ml recombinant human TGF-β1 (R&D systems, Abingdon, UK). Under all experimental conditions, except where indicated, 50 μg/ml ascorbic acid and 100 μg/ml β-aminopropionitrile were added to promote collagen synthesis and prevent cross-links, respectively (both purchased from Sigma). Blocking anti-TGF-β studies were performed by the addition of a neutralizing anti-human TGF-β1, 2, 3 monoclonal antibody (2G7) (20 or 50 μg/ml) [20] or a control mouse IgG at the start of the culture. After 4 days, culture supernatants were harvested, and measured for secreted proteins by enzyme-linked immunosorbent assay (ELISA). Cells were trypsinized and viable cells were counted using Trypan Blue exclusion.

Collagen type I, type III and type IV inhibition ELISA

Collagen type I, type III and type IV accumulation in culture supernatants was measured using a specific inhibition ELISA as previously described for fibronectin [10]. Briefly, 96-well Nunc Maxisorb microtitre plates (Gibco/Invitrogen) were coated with 0.5 μg/ml human collagen type I, type III or type IV (all purchased from Sigma) in phosphate-buffered saline (PBS) (100 μl/well) overnight at room temperature. Collagen type I, type III or type IV standard and samples were pre-incubated overnight at 4°C with the same volume of goat anti-human collagen type I, goat anti-human collagen type III or goat anti-human collagen type IV, respectively (all purchased from Immunoglobulins Direct, Oxfordshire, UK) in PBS/0.05% Tween/2% casein (PTC). Plates were washed and, after a blocking step with PTC for 1 h at 37°C, 100 μl of the pre-incubated standard or sample was added to the plates, and incubated at 37°C for 1 h. After washing the plates, a peroxidase-conjugated secondary rabbit anti-goat IgG (Nordic Immunology, Tilburg, The Netherlands) was added and incubated for 1 h at 37°C. The ELISA was developed with ABTS (2,2’-amino-bis-3-ethylbenzthiazoline-6-sulfonic acid)/H₂O₂ and read at an optical density (OD) of 415 nm.

RNA isolation and semi-quantitative reverse transcription–polymerase chain reaction (RT–PCR)

Total cellular RNA was extracted using RNAzolB (Campro Scientific, Veenendaal, The Netherlands) according to the manufacturer’s description. The quantity and purity of the isolated RNA were measured at OD₂₆₀ and OD₂₈₀ and analysed on a 0.5× TBE (0.045 M Tris borate, 0.001 M EDTA) 1% agarose gel.

For RT–PCR, 1 μg of total RNA was reverse-transcribed into cDNA by oligo(dT) priming using Moloney murine leukemia virus reverse transcriptase (all purchased from Invitrogen, Paisley, UK). cDNA was amplified by PCR using primers for human GAPDH as housekeeping gene (forward primer 5'-ACC ACA GTC CAT GCC ATC AC-3', reverse primer 5'-TCC ACC CTG TTG CTG TA-3', yielding a product of 433 bp) and the α1 subunit of collagen
Collagen accumulation by tubulointerstitial cells in response to hyperglycaemic conditions and TGF-β1

To investigate the accumulation of collagens and their induction in renal cells, fibroblasts and PTECs were cultured in the presence of high ambient glucose, i.e. 25 mM D-glucose. Collagen type I was highly secreted by renal fibroblasts, which was increased further by glucose (Figure 1A). Similarly, the other interstitial collagen (type III) was strongly produced by fibroblasts and increased upon stimulation with 25 mM D-glucose compared with 5.5 mM glucose (normal glucose) (Figure 1B). This increase was not due to the higher osmolarity to which the cells were exposed, since there was no effect on collagen accumulation when cells were exposed to the same concentration of L-glucose. Exposure to the profibrotic growth factor TGF-β1 also resulted in an increase in collagen type I secretion by fibroblasts (Figure 1A). In contrast, collagen type III was not affected by TGF-β1 (Figure 1B). Under no condition was there detectable secretion of the interstitial collagens type I and III by PTECs (Figure 1A and B).

In contrast to the interstitial collagens, the basement membrane component collagen type IV was strongly produced by PTECs, which was increased by exogenous TGF-β1 (Figure 1C). In addition, renal fibroblasts were able to secrete collagen type IV, which was enhanced further by TGF-β1. Upon TGF-β1 stimulation, collagen type IV secretion by fibroblasts reached the same levels as that secreted by PTECs. Importantly, incubations with high glucose resulted in increased

Statistical analysis

The data are presented as means ± SD of triplicate cultures and are representative of at least three independent experiments. Differences between various culture conditions were evaluated by analysis of variance (ANOVA) with a Bonferroni correction for multiple comparison. In some experiments, relative differences were tested with a one-sample t-test. A value of *P < 0.05* was considered to represent a significant difference.

Results

Collagen accumulation by tubulointerstitial cells in response to hyperglycaemic conditions and TGF-β1

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collagen type IV accumulation by fibroblasts (Figure 1C). In our experiments, hyperglycaemic conditions did not result in increased collagen type IV secretion by PTECs, which is different from previously published data with human and murine PTECs [21,22]. To exclude the suggestion that the observed effects were caused by changes in cell number, proliferation of renal fibroblasts was analysed. In contrast to subconfluent cells, quiescent confluent cultures of fibroblasts did not result in significant changes in cell number after exposure to 25 mM d-glucose or TGF-β1 compared with medium control (data not shown). In conclusion, high glucose was able to increase the secretion of all measured collagens by renal fibroblasts, while TGF-β1 was only able to induce specific collagens.

Collagen type IV accumulation in fibroblast cultures from different sources

Since ascorbic acid and β-aminopropionitrile can influence the processing of collagens, we have also investigated collagen type IV accumulation by renal fibroblasts in the absence of these chemicals. Collagen type IV secretion by renal fibroblasts was substantially lower in the absence of ascorbic acid and β-aminopropionitrile (Figure 2A). Nevertheless, stimulation of fibroblasts under these culture conditions with either TGF-β1 or 25 mM d-glucose resulted in increased secretion of collagen type IV.

To investigate whether fibroblasts from different sources can also secrete collagen, type IV fibroblasts derived from a fibrotic kidney (TK188) were used. Collagen type IV was secreted by TK188 cells, and exposure to exogenous TGF-β1 and 25 mM d-glucose resulted in an increased accumulation of collagen type IV (Figure 2B).

To exclude the suggestion that collagen type IV secretion by renal fibroblasts was caused by SV40 transformation of the cells, primary fibroblasts derived from skin were tested. As seen in renal fibroblasts, collagen type IV was secreted by primary fibroblasts, and increased after stimulation with 25 mM d-glucose or TGF-β1 (Figure 2C).

Collagen α1 (IV) chain mRNA expression in fibroblasts

To investigate further the mechanism of collagen type IV expression in renal fibroblasts, collagen α1 (IV) mRNA expression was measured. To quantitate mRNA in different conditions, a semi-quantitative RT–PCR was performed by cDNA titration using GAPDH as housekeeping gene (Figure 3A). Stimulation with TGF-β1 or 25 mM d-glucose for 3 days, but not with 25 mM l-glucose, resulted in the upregulation of collagen α1 (IV) mRNA expression in renal fibroblasts compared with 5.5 mM glucose conditions (Figure 3B). At day 4, TGF-β1-induced collagen α1 (IV) mRNA expression returned to basal levels. Glucose-induced collagen type IV mRNA expression was also lowered compared with 3 day incubations, although it was still significantly increased compared with the normoglycaemic cultures.

Kinetics of collagen type IV secretion by renal fibroblasts

Next, we examined the kinetics of collagen type IV secretion by renal fibroblasts. Stimulation of fibroblasts...
with TGF-β1 resulted in a time-dependent increase in collagen type IV accumulation in culture supernatants (Figure 4A). Compared with the medium control, a significant stimulatory effect was already detectable after 24 h of incubation, reaching a maximum after 2 days of culture (Figure 4B). In contrast, 25 mM D-glucose-induced collagen type IV secretion was delayed: significant increases compared with 5.5 mM glucose were only detectable from day 3 onwards (Figure 4A).

Role of TGF-β1 in glucose-induced collagen type IV production in renal fibroblasts

Since exogenous TGF-β1 stimulated collagen type IV secretion in renal fibroblasts, and TGF-β1 is an intermediate in glucose-induced matrix accumulation by mesangial cells [23], the role of TGF-β1 in glucose-induced responses was investigated. Addition of a neutralizing pan-specific anti-TGF-β antibody completely inhibited the stimulatory effect of 1 ng/ml TGF-β1 on the secretion of collagen type IV by fibroblasts, while a control antibody had no effect (Figure 5, left panel). In contrast, this neutralizing antibody could not attenuate the stimulatory effect of high ambient glucose on the secretion of collagen type IV by renal fibroblasts (Figure 5, right panel).

Discussion

In the present study, we have investigated the production profile of interstitial and basement membrane collagens by renal fibroblasts and PTECs and their induction under hyperglycaemic conditions. We demonstrate that fibroblasts not only secrete the interstitial components collagen type I and III, but also the typical basement membrane component collagen type IV. In contrast, PTECs were only able to secrete collagen type IV. Importantly, we found that secretion of collagen type IV by renal fibroblasts was increased...
Regulation of collagen type IV secretion by renal fibroblasts

In tubulointerstitial fibrosis, the composition of the interstitium is altered, including increased presence of collagen type I, III and IV [13]. Here, we demonstrate that, while high glucose was able to increase both collagen type I and type III in renal fibroblasts, TGF-β1 only increased collagen type I secretion. Under no condition was there increased secretion of interstitial collagens by PTECs. In addition to quantitative changes in collagens, expression of novel matrix components in the tubulointerstitium during fibrosis has also been observed: the expression of collagen type IV is no longer restricted to the basement membranes, but is also expressed in the interstitium [8,11,12,14,15]. The aberrant expression of collagen type IV in the interstitium during fibrosis has been attributed predominantly to TECs [8]. Only a few studies have examined the expression of collagen type IV in renal fibroblasts [17], and there is still little known about its regulation and the relative contribution of renal fibroblasts and TECs to the aberrant expression of collagen type IV during fibrosis. We now confirm that human renal fibroblasts secrete collagen type IV. Although basal collagen type IV secretion by fibroblasts was low compared with PTECs, the secretion of collagen type IV by fibroblasts was increased after stimulation with TGF-β1 and high glucose, reaching levels comparable with those secreted by PTECs. Therefore, we suggest that as well as PTECs, fibroblasts can also contribute to the excessive and aberrant collagen type IV deposition in the renal interstitium.

In our hands, using HK2 cells, exposure to high glucose did not result in an increased collagen type IV production. This is in clear contrast to previous studies with human and murine PTECs [21,22] and might be a specific feature of this cell line. Since our standard protocol to generate primary human PTECs uses DMEM/HAM F-12 which already contains high glucose (17.5 mM), we have not been able to address this question specifically.

The increase in collagen type IV protein secretion was accompanied by an increase in collagen α1 (IV) gene transcription in renal fibroblasts. However, after an initial increase in collagen α1 (IV) mRNA expression by high glucose, the expression levels were already falling after 4 days of incubation. These data seem to conflict with our kinetics study which showed a concomitant continued rise in collagen type IV protein secretion. However, ECM turnover is regulated by synthesis and degradation of matrix components. It is thought that disruption of the balance between synthesis and degradation leads to uncontrolled ECM turnover [24]. Therefore, it is feasible that glucose-induced deposition of ECM is accomplished by an initial increase in ECM synthesis accompanied by increased ECM transcription. Subsequently, ECM accumulation is maintained by a decrease in ECM proteolysis.

One of the most potent mediators of fibrosis is undoubtedly TGF-β. It has been shown that TGF-β plays a central role in the development of diabetic nephropathy, and numerous studies have demonstrated the close resemblance of the effect of TGF-β and high ambient glucose in the synthesis of ECM components [19]. Previous studies by our group and others, especially in mesangial cells, have demonstrated that glucose-induced effects were mediated by TGF-β1 [23]. However, several studies have suggested the existence
of TGF-β-independent pathways. For instance, it has been demonstrated that the expression of the α1 and α5 subunits of collagen type IV induced by glucose is independent of TGF-β1 in mouse podocytes [25]. We now show that glucose-induced collagen type IV secretion in renal fibroblasts is also independent of endogenous TGF-β, comparable with our previous findings with fibronectin [10]. Thus, in addition to the interstitial matrix components, basement membrane proteins are also regulated by glucose via a TGF-β-independent pathway in human renal fibroblasts. These data suggest that the regulation of matrix metabolism under hyperglycaemic conditions is different for renal fibroblasts compared with other renal cell types.

An important question is which alternative factors might be responsible for the observed increased ECM expression in renal fibroblasts. Although direct effects of glucose cannot be excluded, the pronounced delay in kinetics does suggest production of autocrine mediators. Attractive candidates to mediate glucose-induced regulation of ECM in our culture system are angiogenins II, insulin-like growth factor-I and connective tissue growth factor, since these have been implicated as TGF-β1-independent inducers of ECM [26–28].

In conclusion, we have shown that renal fibroblasts, in addition to the interstitial components collagen type I and III, also secrete collagen type IV. Stimulation with glucose resulted in increased secretion of collagen type IV, which was independent of endogenous TGF-β. These data suggest that renal fibroblasts can contribute to the increased expression of collagen type IV in tubulointerstitial fibrosis observed during diabetic nephropathy.

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Conflict of interest statement. None declared.

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