Abstract Mesangial cells are responsible for the synthesis of mesangial matrix as well as its degradation, which is mediated by a number of proteolytic activities, including metalloproteinases (MMPs). Imbalanced matrix protein metabolism may be responsible for mesangial expansion and glomerulosclerosis in diabetic nephropathy. Heparin prevents this complication. In human and murine mesangial cell cultures, RT-PCR was able to detect mRNA expression for a number of molecules involved in the mesangial extracellular matrix turnover: type IV collagen (α1(IV)COLL), MMP-1, MMP-2, MMP-3, MMP-9 and MMP-10, and the tissue inhibitors TIMP-1 and TIMP-2. The expression of mRNA for α1(IV)COLL and MMP-2/TIMP-2 balance was studied in human cells in the presence of high glucose and heparin. RNAs for all the studied molecules were expressed at different levels. Interestingly, a shift in the balance of α1(IV)COLL, MMP-2 and TIMP-2 was observed in high glucose, which was partially reversed by heparin supplementation. The new equilibrium was mostly due to the down-regulation of type IV collagen expression, rather than further reduction of potential proteolysis. Our data, while extending the list of potential mediators of mesangial matrix catabolism, highlight a molecular mechanism by which the pathogenesis of diabetic nephropathy may be sustained, and at the same time suggest that heparin may have the potential to correct this abnormality.

Key words: α1(IV)COLL; glucose; heparin; mesangium; MMP-2; TIMP-2

Introduction

Regulation of mesangial matrix is a dynamic process involving synthetic as well as degradative processes. The latter involve a number of matrix metalloproteinases (MMPs) [1]. Recently, the issue of MMP activity in diabetic nephropathy has been addressed, and the idea proposed of a reduced degradation as a possible mechanism leading to enlargement of the mesangium [2]. Although this hypothesis may well be valid, the published data, resulting from different model systems and methodological approaches, are contradictory as regards which proteases are influenced by diabetes and the mechanism involved. Very little is known about the effect of diabetes on MMP modulation by their tissue inhibitors (TIMPs), and in particular by TIMP-2, the most specific inhibitor of the 72 kDa type IV collagenase (MMP-2). Furthermore, the available data do not match the evidence that in experimental diabetic nephropathy one of the earliest changes in mesangial matrix is the increased expression and synthesis of α1(IV) collagen chain [α1(IV)COLL] [3].

We have recently described the effect of heparin treatment in the streptozotocine diabetic model [4]; heparin induced a reduction of matrix, specifically of the α1(IV)COLL. Our data indirectly suggested a possible effect of heparin not only at the level of collagen synthesis, but interestingly on degradation as well. However, in smooth muscle cell cultures, heparin left unmodified the expression of MMP-2, which specifically degrades type IV collagen, while lowering the expression of MMP-3 (stromelysin-1), MMP-9 (92 kDa type IV collagenase), tissue-type plasminogen activator, and MMP-1 (interstitial collagenase) [5].

This study has been carried out as a first-step investigation, before moving on to in vivo animal models, to evaluate the mRNA expression of a number of important MMPs and TIMPs, and the effect of high glucose concentration and heparin on the α1(IV)COLL and MMP-2/TIMP-2 mRNA balance expressed by mesangial cells.

Subjects and methods

Cell cultures

Cultures of human mesangial cells (HMCs), donated by P. Mené (Rome, Italy), were maintained in RPMI-1640
medium (Gibco, UK), which contains 11 mM glucose with 10% FCS, 100 µg/ml penicillin, 100 µg/ml streptomycin, and 2.5 mM fungizom. Between passage 10 and 14, 3 × 10^6 cells were seeded in a 225 cm² flask (Bibby Sterilin Ltd, UK) and cultured for 8 days with and without 100 µg/ml LMW heparin (Fluxum, Alfa Wassermann SpA, Italy), 30 mM glucose (final concentration), or 100 µg/ml LMW heparin plus 30 mM glucose, at 37°C and 5% CO₂. The culture medium was renewed every 2 days. Primary and SV40 transgenic murine cells (MMCs) (ATCC) were obtained and grown as described [6].

**RNA extraction**

At the end of incubation, the cells were rinsed with PBS without calcium and magnesium, trypsinized and pelleted. The total RNA was extracted from cellular pellet using the RNeasy kit for RNA extraction (QIAGEN, Italy), RNA yield and purity were measured by spectrophotometric determinations at 260 and 280 nm.

**cDNA synthesis**

Reverse transcription of 2.5 mg total RNA using 0.2 µg oligo-dT primer was performed for 1 h at 42°C using 25 U of AMV reverse transcriptase (Boehringer Mannheim, Italy) in 50 mM Tris (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 1 U RNasin (Promega Corporation, Madison, USA) and 1 mM each of dATP, dGTP, dCTP, and dTTP. For removal of RNA-cDNA hybrids, produced by first-strand cDNA synthesis, 1 U of ribonuclease H (Pharmacia, Uppsala, Sweden) was added and the mixture incubated for 1 h at 37°C. The cDNA pool was serially diluted in water from 1:5 to 1:625. The 1:5 dilution corresponded to 500 ng RNA; hereafter, when a quantity of RNA is indicated as being used in the polymerase chain reaction (PCR), this refers to the generated cDNA derived from the quoted RNA amount. Each dilution was aliquoted in amplification tubes and stored at −20°C until PCR.

**PCR**

The 50-µl reaction volumes contained the following: 20 µl of appropriate cDNA dilution, Taq DNA polymerase (1.25 U; Pharmacia), dNTPs (0.2 mM each of dTTP, dCTP, dGTP, dATP; Pharmacia) and oligonucleotide primers (0.5 µM each), made up in a final concentration of 1 × PCR reaction buffer (10 mM Tris–HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl) and overlaid with mineral oil. Amplification was performed in sequential cycles with 2 min denaturation at 94°C, 1 min primer annealing at 56°C and 2 min extension at 72°C; after the last cycle, all samples were incubated for an additional 10 min at 72°C. To study the constitutive MMP and TIMP mRNA expression panel in the mesangial cell model systems, amplifications were carried out for 40 cycles. The comparative analysis under glucose and heparin effects, a non-radioactive method was used for amplicon detection: after gel separation, DNA was transferred onto a nylon membrane (Hybond N +, Amersham Italia Srl, Italy) by means of alkaline blotting. Specific DNA probes for MMP-2, TIMP-2, α(IV)COLL and β₂-microglobulin were obtained by PCR amplifying the cDNA of the HT-1080 human fibrosarcoma cell line, and the respective sequences were verified by theideoxynucleotide chain termination method. Probe labelling, membrane hybridization and signal detection were performed by ECL Direct System (Amersham) following the manufacturer’s instructions. Autoradiograms were analysed by scanning densitometry using a Hoefer GS 300 densitometer (Hoefer, San Francisco, California). Areas under the curve were calculated by means of software supplied by Hoefer. Comparative analysis was performed for each species on point fitting into the linear range of amplification (8 ng RNA). MMP-2, TIMP-2 and α(IV)COLL values from control and treated cultures were then ‘normalized’ to the corresponding β₂-microglobulin expression (MMP-2/β₂-microglobulin, etc.; values are then independent from different loading). To evaluate possible fluctuations of the MMP-2/TIMP-2 balance between different culture conditions, the results were expressed using the following formula which, taking into account variations in the expression of both the enzyme and its inhibitor, describes any imbalance in favour of either MMP-2 or TIMP-2 under the experimental conditions [8]:

\[
\frac{\text{MMP-2}}{\text{TIMP-2}} = \frac{\text{MMP-2}_{\text{treated}}}{\text{TIMP-2}_{\text{control}}} \cdot \frac{\text{MMP-2}_{\text{control}}}{\text{TIMP-2}_{\text{treated}}}
\]
Statistical analysis

One-way analysis of variance and Bonferroni’s test for multiple comparisons were applied to the results obtained from quadruplicate experiments.

Results

MMPs and TIMPs RT-PCR panel in mesangial cells

The human (HMCs) and murine (MMCs) mesangial cell models were characterized for metalloproteinase and tissue inhibitor mRNA expression. Although quantitative differences were not studied, all tested markers were constitutively expressed at levels whose amplicons were well detectable after 40 cycles and electrophoresis in agarose gel followed by EtBr staining. Figure 1 shows the amplification products for MMP-1, MMP-2, MMP-3, MMP-9, TIMP-1 and TIMP-2 in HMCs. In MMCs the results were equivalent, but MMP-10 substitutes MMP-3 (not shown). The expression of α1(IV)COLL mRNA was also confirmed in HMCs (see below). The results, showing unique bands for each amplicon, highlight the specificity of the chosen primers and the optimization of the PCR procedure [8].

In order to evaluate possible variations in the expression of the mRNAs under different experimental conditions, the range over which PCR amplification was linear was determined by preliminary experiments: Fig. 2 shows that, for each target molecule, linearity is maintained between 5 and 25 ng of corresponding RNA. Following results were obtained in four separate experiments of which Fig. 3 shows an example. Mean values and standard deviations are reported in Fig. 4.

Effect of glucose and heparin on type IV collagen mRNA expression in HMCs

HMCs showed consistently lower α1(IV)COLL mRNA expression when cultured in the presence of both 30 mM glucose and 100 ng/ml heparin (F = 43.38; P = 0.000), compared to cultures in the presence of each separately (P < 0.001 for both). An example is shown in Fig. 3. The comparative quantitation of the amplicons showed no statistical differences, compared to the control, when glucose and heparin were separately supplemented to the cells (Fig. 3). Conversely, when the cells were cultured in the presence of both glucose and heparin, their expression level fell consistently by 71%.

Effect of glucose and heparin on MMP-2 and TIMP-2 mRNA expression in HMCs

All treatments (+ glucose, + heparin, + glucose + heparin) significantly reduced MMP-2 mRNA expression compared to control cells (P < 0.001 for all comparisons), although by different amounts (−68%, −29% and −74%, respectively; F = 44.63, P = 0.000) (Figs. 3 and 4). In the case of TIMP-2, glucose alone or in association with heparin reduced the inhibitor mRNA expression by 27% and 36%, respectively (P < 0.001 for both comparisons), while a slightly non-significant increase was registered in the case of heparin alone (F = 27.89, P = 0.000). In order to have insights about the potential net proteolytic activity under different experimental conditions (although being conscious that messenger quantitation and enzymatic activity are not necessarily coincident), the MMP-2/TIMP-2 mRNA balance was calculated. As shown in Fig. 4, all treatments consistently reduced the protease/inhibitor balance (F = 61.68, P = 0.000): glucose the least (−33%), and glucose with heparin the most (−70%).

Discussion

In diabetic nephropathy, the expansion of the mesangium is closely related to the prognosis of diabetic patients, since increased mesangial area is predictive of kidney failure. It is generally considered that mesangial expansion in diabetes is due to the extracellular matrix (ECM) enlargement rather than to mesangial cell hyperplasia. Indeed, increased matrix synthesis has been reported both in vivo in diabetic animals, and in vitro in mesangial cell cultures in high glucose concentrations, or in the presence of advanced glycosylation end products; many of these phenomena have been related to protein kinase C (PKC) and/or TGFβ activation [17,18]. Increased synthesis of type I, III and IV collagens, laminin and fibronectin have been reported; in particular, the enhanced production of collagen is a central event in diabetic glomerular ECM abnormalities, type IV collagen being one of the first matrix proteins involved [19]. However, matrix regulation is a dynamic process that also involves degradation. The glomerular MMPs are probably the most important proteinases in the degradation of mesangial matrix, and many have been described.

We now report the expression of a number of MMP mRNAs in primary HMCs (Fig. 1), and primary and SV40-transgenic MMCs together with the correspond-
Fig. 2. Linear range-finding experiments: cDNA corresponding to 250 ng RNA was serially diluted 1:2 and PCR amplified using primers specific for each molecule and fixed reaction conditions.

In regenerating tissue inhibitors, TIMPs. The expression of MMP-2 and TIMP-2, which balance has been documented crucial in type IV collagen catabolism [8], is in accordance with previous reports: in fact, both have been described by biochemical and molecular biology methods in HMC cultures and in human glomeruli [20]. Our data confirm the expression of MMP-2 and TIMP-2 in HMCs and demonstrate their expression in MMCs as well. As far as MMP-9 is concerned, its expression has been only occasionally observed usually after stimulation [20], and never described in MMCs. This paper reports the expression of MMP-9 in both HMCs and MMCs cultured in basal conditions. Also the expression of MMP-1 and MMP-10 is described, contrary to a previous report on human mesangial cells [20], but confirming Carome’s data with regards to the latter [21]. Finally, TIMP-1 mRNA expression in cell lines from both species confirms previous reports. Detection of mRNA in primary and immortal lines suggests that the expression of all these molecules is a specific feature of these cells.

Inden, interest is growing concerning the role of matrix degradation in diabetic nephropathy, and the hypothesis has been forwarded that in its pathogenesis an important role is played by less efficient degradative matrix processes due to the diabetic milieu. An early reduction of glomerular MMPs has been documented in rat streptozotocin (STZ)-induced diabetes [2], and a 117 kDa MMP has been reported to be most involved. In vitro elevated glucose in the media of cultured rat mesangial cells causes suppression of the expression of transin, a metalloprotease in rats similar to stromelysin [22], which has a broad spectrum of substrates but with a much lower affinity to type IV collagen than MMP-2 [1].

Mesangial cells constitutively express MMP-2, which specifically degrades type IV collagen, its inhibitor TIMP-2 and α1(IV), which is mesangial matrix-specific type IV collagen chain [20, present data]. Therefore, mesangial cells have the potential to completely modulate the synthesis and degradation of the most important component of mesangial matrix. We believe it is
Glucose and heparin on \( \alpha(IV) \)COLL, MMP-2 and TIMP-2 in mesangial cells

For this reason, we have focused attention on the effect of high glucose, a condition simulating the diabetic milieu, on the balance between the expression of \( \alpha(IV) \)COLL, MMP-2 and TIMP-2. Furthermore, we were also interested to evaluate the effect of heparin because of its recently described effect on diabetic nephropathy [4].

Glucose had no significant effect on collagen mRNA, a result which at first glance might appear surprising in view of *in vivo* and *in vitro* reports showing that, in an animal model of diabetes mellitus and in mesangial cell cultures in high glucose, type IV collagen gene is up-regulated [23,24]. However, the glucose-dependent collagen up-regulation has been observed in short-term mesangial cultures, reaching a plateau on the third day and lasting until at least the seventh [24]. Nevertheless, Silbiger *et al.* [25] showed that in long-term (2–4 weeks) culture conditions, without tripsinization, type IV collagen gene expression is not changed. These results, with which the present data conform, have been interpreted as the effect of non-enzymatic glycation of mesangial matrix, but direct transfer of the *in vitro* results to *in vivo* conditions must be cautiously considered.

Heparin alone does not modify the expression of type IV collagen in mesangial cells, as described in smooth muscle cells [26] and observed in the diabetic rat [23]. However, in the presence of high glucose concentration, heparin dramatically down-regulates the type IV collagen gene, a result that resembles *in vivo* activity [4]. Both high glucose concentration and heparin, alone or together, reduced mesangial cell MMP-2 mRNA with a moderate synergistic effect. While TIMP-2 gene expression was unaffected by heparin, and moderately down-regulated by glucose, it appeared consistently reduced by the combination of the two (Fig. 4).

As a whole, these data may reflect an unbalanced equilibrium between synthesis and degradation of type IV collagen when mesangial cells are grown either in high glucose or in the presence of heparin, with potentially higher steady-state level of collagen. Although at lower expression level, a new balance between synthesis and degradation is conversely restored when cells are incubated with both, which may better parallel that of control cells. The new equilibrium is mostly due to the down-regulation of type IV collagen expression, rather than further reduction of potential proteolysis.

A number of MMPs have been demonstrated to be inducible by phorbol ester (PMA), at least in smooth muscle cell cultures [5]. The induction of these MMPs is also inhibited by staurosporine and by heparin after pretreatment with PMA, supporting the involvement of the PKC cascade in MMP expression. It is worth noting that both high glucose and heparin may interfere with PKC: high glucose concentration is known to increase PKC activity in mesangial cells [27], and heparin inhibits this pathway downstream of PKC.
References

16. Edwards DR, Waterhouse P, Holman ML, Denhardt DT. A growth-responsive gene (16CSY) in normal mouse fibroblasts is modulated by heparin and other glomerular diseases’ is also acknowledged. Human Nephrology, La Sapienza University of Rome. We are grateful to
17. Acknowledgements. This research was supported by grants from CNR-ACRO and CNR-Bilaterale, Associazione Italiana per la Ricerca sul Cancro, and MJRST, Italy. The support of the European Economic Community through BIOMED-1 ‘Concerted action on alterations of extracellular matrix components in diabetic nephropathy and other glomerular diseases’ is also acknowledged. Human mesangial cell cultures: molecular clues for its activity in nephropathy. Nephrology, La Sapienza University of Rome. We are grateful to Dr Susan Biggin for revision of the English manuscript.

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