ROLE OF METALLOPROTEASES AND INHIBITORS IN THE OCCURRENCE AND PROGRESSION OF DIABETIC RENAL LESIONS

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SUMMARY - Renal remodelling in hyperinsulinaemic/insulinopenic states is mediated by glaucotoxicity, endothelial dysfunction and vascular and nephron collagen turnover. Hypertensive and renal links are re-NEWED by renoprotective interventions of renin-angiotensin. Vasoactive peptide processing and vascular collagen deposition are under the tight control of two zinc metalloproteinase families that regulate vascular tone and trophicity: gluzincins (or vasopeptidases) are convertases of angiotensins, endorphins or atrial natriuretic factors; and metzincins (or matrix metalloproteases [MMP, matrixins]) regulate vascular type IV collagen basement membrane proteolysis. Association of natural tissue inhibitors of MMPs, pharmacological inhibitors of vasopeptidases (either conventional [angiotensin-converting enzyme inhibitors] or innovative [omapatrilat]), together with synthetic MMP inhibitors, are currently screened to counteract vascular remodelling and renal scarring. Our studies focused on the 72 kDa (MMP-2) and 92 kDa (MMP-9) matrix gelatinases and tissue inhibitors involved in basement membrane degradation and re-building. Three complementary settings were developed, allowing evaluations from basic to clinical stages. A leucocyte-endothelial transmigration model was designed for transcription and addressing of enzymes and inhibitors, in situ matrix degradation, and blocking by metalloprotease inhibitors (captopril). Insulin-resistant fructose-fed rats showed heavy proteinuria and glomerulosclerosis involving angiotensin II-dependent changes in renal gelatinases and inhibitors. Urinary gelatinolytic profiles from Type 2 diabetic patients with overt nephropathy were compared with those of normal first-degree relatives and age-matched healthy controls. Physiologically, MMP-9 was the primary urinary gelatinolytic enzyme. In Type 2 diabetic proteinuric patients, MMP-9 and MMP-2 releases were significantly increased in the absence of renin-angiotensin blockade, while first-degree relatives showed reduced gelatinase levels suggestive of a genetic control of renal matrix regulation prior to potential glaucotoxic dysregulation. These preliminary data suggest that local MMP/TIMP imbalance is involved in diabetic renal remodelling. Further studies are needed to define the redundancies and specificities of vasopeptidase and MMP inhibitors, differentiate the anti-hypertensive effect from target-organ protection, screen for innovative pharmacological compounds, and validate simple, efficient biological markers of renal fibrosis progression and the effect of anti-fibrotic therapeutic interventions.

Key-words: diabetic nephropathies, matrix metalloproteinases, TIMPs, vasoactive peptidases, synthetic inhibitors.

RÉSUMÉ - Métalloprotéases et inhibiteurs dans la constitution des lésions rénales du diabète.

Le rein est une cible du remodelage tissulaire lors d’insulinothérapie. Les relations de la glaucotoxicité (fibroblastes interstitiels, méésangium, radicu- libres, cytokines, TGFi) participent aux remaniements des parois vasculaires et des néphrons. Les liens entre hypertension et maladies rénales ont été reconsidérés grâce aux interventions sur le système rénine-angiotensine dans la néphropathie des diabètes complétés d’une analyse du clivage, de la mise à disposition et de la régulation des peptides vaso-actifs et structuraux des parois de la microcirculation. L’équilibre entre la mise à disposition de peptides vaso-actifs et la régulation des dépôtés de collagène fait appel à deux familles de protéases à zinc qui sont des déterminants majeurs du tonus et de la trophicité vasculaire, donc des débits et de l’irrigation tissulaire : les gluzincines, vasopepti- des, dont les convertases de l’enzyme de conversion, des endothélines ou du facteur atrial natriurétique; les metzincines ou métalloprotéases matricielles (MMP) régulent la protéolyse des composants structuraux (collagènes IV). Aux inhibiteurs physiologiques des MMP (TIMPs) s’ajou- tent les inhibiteurs des vasopeptidases, conventionnels (IEC) ou inno- vants (omapatrilat), et des inhibiteurs de MMP en cours de développe- ment. Nous avons développé les méthodes d’analyse de la régulation des deux enzymes de dégradation des basales vasculaires appartenant à la famille des MMP, les gelatinases de 72 kDa (MMP-2) et de 92 kDa (MMP- 9), et mis en évidence l’effet anti-inflammatoire et anti-fibrosant du cap- topril dans un modèle in vitro de transmigration endothéliale des leuco- cytes. En plus de son effet inhibiteur de gluzincines comme l’enzyme de conversion de l’angiotensine, le captopril inhibe l’activité des gelatinases sécrétées en tant que piégeur de zinc et inactive le TIMP-1. Le modèle de transmigration endothéliale permet d’analyser la transcription et l’adressage des enzymes et des inhibiteurs et de caractériser l’état des matri- ces extracellulaires. L’âtème rénale du modèle de glomerulosclérose du rat fructose insulinorésistant associe protéinurie, hypertrophie ré- nale, glomerulosclérose et hyalinose vasculaire, et fait intervenir les deux gelatinases et leurs inhibiteurs tissulaires.


Mots-clés : gluzincines, metzincines, gelatinases, inhibiteurs tissulaire- res TIMPs, inhibiteurs pharmacologiques, peptidases vasoactives, fi- brose rénale, collagènes, néphropathie diabétique.

The epidemiology of renal diseases affecting the course of Type 2 diabetes is evolving in Western countries. In France, an increased incidence (7 to 12%) of renal replacement therapies was noted between 1989 and 1995 (Uremidiab 2) [1]. Unfavourable outcomes and the cost of complications indicate that early screening and intervention are needed. The distinctive signs of renal disease, mainly increased urinary albumin excretion rates and loss of glomerular filtration rate (GFR), are preceded by early changes in cell-matrix interaction. This study focused on determinants of renal fibrosis and the role of matrix-degrading enzymes in initiation of collagen neosynthesis [2].

**BACKGROUND**

Insulin resistance/insulinopenia is conducive to several pathways of macro-/microvascular endothelial remodelling [3]. Glucotoxicity, glycation, gene regulation responses to inflammatory, oxidative, or mechanical shear stress, and balanced vasoactive mediators (angiotensin II, ET-1, nitric oxide), are induced by a first wave of immediate early gene transcription (c-fos, c-jun, sp-1, NF-KB), which controls the expression of mediators of cell-cell interactions (cytokines, matrix-degrading enzymes, and inhibitors) [4-6]. The final common response integrates coordinate changes in cell behaviour (secretion, proliferation, apoptosis) and extracellular matrix remodelling.

The heterogeneity of renal lesions in Type 2 diabetes (Fioretto, Cordonnier) [1] requires sequential renal biopsy protocols to assess initial histological levels and tissular outcomes of therapeutic interventions. Early changes require ultrastructural quantitative morphometric measurements of sectorial volumes. Ethical limitations and the variable rates of structural changes imply a search for non-invasive urinary biochemical markers related to the theoretical mechanisms of disease progression. In this setting, diabetic nephropathy, as a non-inflammatory vascular extracellular deposit disease, appears to be a good model for evaluation of collagen IV turnover. Changes in rates of reticular collagen (types IV and V) synthesis and induction of non-reticular collagens I and III have been observed by several groups (Esposito, Rossert) [7,8] in relation to functional disturbances in glomerular basement membrane (GBM). Collagen degradation results from the synthesis and activity of matrix metalloproteinases (MMPs, matrixins) and co-secreted tissue inhibitors [2,9,10]. This alternate pathway for disease evaluation includes other metalloproteinases such as vasopeptidases [11] [angiotensin-converting enzyme (ACE) since renin-angiotensin blockade has a major impact on renal survival. MMP regulation [9] includes the release of inactive pro-enzymes, pericellular activation, formation of soluble complexes with tissue inhibitors of metalloproteinases (TIMPs) and with un-specific inhibitors (2-macroglubulin, Tamm-Horstell protein) or ligand binding with N-Gal (lipocalin) or collagen IV fragments. New data on specific MMPs acting on collagen IV, expressed as reactive intermediates of cell activation, potentially induced by the hyperglycaemic environment and inhibited by synthetic inhibitors, prompted us to evaluate 72 kDa and 92 kDa gelatinases, respectively MMP-2 and MMP-9 [12,13], in three complementary outcomes (Fig. 1):

- a cellular *in vitro* model of leucocyte-endothelial transmigration was built to observe changes in enzymes and surrounding matrices during cell activation and to quantify the effect of synthetic inhibitors;
- an experimental nutritional model of glomerulosclerosis and proteinuria close to human syndrome X in fructose-fed rats was also induced to qualify ageing changes and therapeutic actions on insulin resistance (metformin) and the renin-angiotensin axis;
- preliminary clinical studies were intended to define baseline urinary gelatinase levels and pre/posttherapeutic changes as tools for target-organ damage screening and biological assessment of interventions.

**EVALUATION LEVELS**

**Leukocyte – endothelial transmigration**

The use of endothelial monolayers set over synthetic matrices (Matrigel®, gelatin-agar) on the bottom side of filters allowed us to reconstitute *in vitro* an integral barrier to leucocyte transmigration (Fig. 2) [14]. Activation by chemoattractants (IL-8) or increasing glucose concentrations in the lower compartment induced rapid transmigration and enhanced dextran permeability, together with high levels of secreted neutrophil 92 kDa gelatinase (33.5 ± 9.2 µg/ml) ten times greater than those of its natural inhibitor, TIMP-1. Activated endothelial layers expressed and activated a 72 kDa gelatinase (MMP-2) and endothe-
lial MMP-9 (1.5 ± 0.7 µg/ml), TIMP-1 and TIMP-2, but at levels far below the matrix proteolytic potential of marginating leucocytes [15]. Enzyme activities and transmigration processes were drastically and similarly reduced by specific anti-92 kDa gelatinase antibodies, synthetic MMP inhibitors, recombinant TIMP-1 and ACE inhibitors (captopril). Immuno-histochemistry and immunogold labelling of endothelial monolayers and neutrophils confirmed the specific targeting of 92 kDa MMP-9 and TIMP-1 to plasma membranes and cell surroundings [16] inducing gelatinase-dependent degradation of basement artificial matrices (as analysed by in situ zymography).

**Fructose-fed rats**

Sugar substitutes could represent an interesting diabetic diet since oral intake of fructose induces mild hyperglycaemia, with reduced secondary insulin secretion. However, chronic side effects of fructose-rich diets (insulin resistance, hyperuricaemia, hypertriglyceridaemia) are related to the clinical dilemma of glucose intolerance, systemic hypertension and renal lesions, which is suspected in human metabolic syndrome X and can lead to full-blown Type 2 diabetes [17].

We studied a cohort of 42 Wistar littersmates allocated to three different intervention protocols: group C (control) (n = 12) was fed with standard diet chow; group F (fructose) (n = 21) was provided with a 58% fructose-enriched diet; and group FM (fructose-metformin) (n = 9) received simultaneously a 58% fructose-enriched diet and an oral stimulus of insulin secretion (metformin). Animals were followed up during ageing (11 to 17 months). Glucose intolerance was confirmed by an awake euglycaemic clamp technique. Renal, metabolic and haemodynamic assessments were obtained at baseline and during angiotensin II infusion with and without angiotensin II receptor blockade. Organomegalay assessment, histology of target tissues and glomerular cultures, and extractions were performed at the time of sacrifice. Our fructose model resembled human metabolic syndrome X since it associated insulin resistance and hypertriglyceridaemia in the absence of obesity, hypertension, and fasting hyperglycaemia, but did not include constituted atherosclerosis or retinopathy. Hypertriglyceridaemia in group F (3.6 ± 1.2 g/l) was significant compared to group C (2.4 ± 1.9 g/l; p < 0.001) and normalised by metformin.

The major changes concerned renal findings, which showed permanently elevated urinary albumin excretion rates in group F (360 ± 65 mg/l) vs group C (84 ± 37 mg/l; p < 0.001), with coordinate increases in urinary and renal gelatinases in the F and FM groups (Fig. 3). More interestingly, sequential assessment of MMP-2 and MMP-9 in cortical, glomerular, tubular and urinary extracts showed increasing release of gelatinases along the nephron segments, suggesting that epithelial cells were the major contributors to matrix-degrading enzyme production. Focal renal lesions associated glomerular mesangium thickening (without typical nodular deposits of diabetic glomerulosclerosis), vascular hyalinosis and areas of ischaemic tubulo-interstitial fibrosis. Areas of glomerulosclerosis...
and tubular atrophy were concurrently stained with anti-MMP9 and anti-smooth muscle cell α-actin, a marker of myofibroblast differentiation. Urinary albumin excretion rates, creatinuria and renal gelatinases were markedly increased by angiotensin II infusion (significantly more in fructose-fed than control animals). Angiotensin II type 1 receptor blockade reduced baseline and angiotensin-induced proteinuria as well as elevated urinary gelatinase release. It is unclear whether these results were due to glomerular haemodynamic changes, the growth factor effect of angiotensin II or leucocyte margination within kidneys.

The fructose rat model appears to be a promising means of defining the effects of the glucose alternative pathway and observing the consequences in vivo of insulin resistance and glomerular hyperfiltration before or in the absence of systemic hypertension, atherosclerosis, or a hyperglycaemic environment. It allows the characterisation of the functional and tissue impacts of interventions on insulin secretion or the renin-angiotensin cascade over targeted end-points of basement membrane remodelling, such as matrix metalloproteinases and inhibitors.

**CLINICAL STUDIES IN TYPE 2 DIABETIC PROTEINURIC PATIENTS**

There is a lack of validated biological markers for therapies intended to correct elevated blood pressure, persistent microalbuminuria, and a fall in GFR over time and thus prevent end-stage renal failure.

As renal gelatinases and in situ collagen IV remodelling are difficult to assess directly [18], urinary profiles of both gelatinases were evaluated in a randomised, prospective, double-blind, one-year follow-up pilot study. Thirteen hypertensive Type 2 diabetic patients with abnormal urinary albumin excretion (UAE) [range 54-1,682 µg/min] and subnormal GFR [Inulin clearance 108 ± 29 ml/min/1.73 m²] were enrolled. After wash-out of calcium blockers and ACEI, patients were randomly allocated either to amlopidine 5 mg or captopril 50 mg twice daily. Urinary gelatinases were monitored in morning urine samples by quantitative densitometry scanning of gelatin zymograms and ELISA. Normal control values were determined from 16 age-matched normal individuals with normal UAE rates below 20 µg/min. A third-party assessment was conducted in 8 first-degree relatives of Type 2 diabetic nephropathic patients over 40 years of age and with UAE rates below 20 µg/min. Preliminary results at study initiation suggested a net increase in urinary 92 kDa gelatinase release over TIMPs (independently of UAE) in baseline levels of Type 2 diabetes (latent MMP-9: 1,238 ± 781 ng/mmol creatinine) compared with normal controls (47 ± 9 ng/mmol creatinine; p = 0.001) and first-degree relatives (0.02 ± 0.01 ng/mmol creatinine; p = 0.0005 vs normal controls). Latent and active MMP-2 levels showed the same significant trends, but with reduced ranges. Latent urinary MMP-2 in Type 2 diabetes reached 115 ± 34 ng/mmol creatinine (p = 0.018 vs controls) compared to control MMP-2 (19 ± 12 ng/mmol creatinine) and MMP-2 of first-degree relatives: 3.3 ± 3.9 ng/mmol creatinine (p = 0.001 vs controls). 92 kDa gelatinase excretion decreased significantly vs baseline after three months of BP control with a CCB or an ACEI, without any significant changes in BP, GFR and PAH clearances and HbA1C. The values at the end of this one-year survey are currently being analysed.

Diabetic urine 92 kDa gelatinase appears to be a novel independent index of renal involvement in clinical studies of Type 2 diabetic patients. The use of CCB or ACEI markedly reduces these abnormalities at identical blood pressure (BP) and GFR levels. This preliminary finding reinforces specific nephroprotective therapies, but will need confirmation at the end of the study. The findings in first-degree relatives of Type 2 diabetic patients could be indicative of genetic abnormalities of matrix remodelling or early metabolic alterations prior to microalbuminuria. The future determination of the metabolic phenotypes of first-degree relatives of Type 2 diabetic patients, such as waist-hip and glucose-insulin ratios, C-peptide secretion and lipid profiles, would help confirm whether reduced urinary gelatinase rates are early markers of metabolic and renal disturbances prior to an increase in UAE levels.

**CONCLUSION – PERSPECTIVES**

The involvement of disturbed collagen IV metabolism in macro- (atheroma) and microvascular remodelling (tissue fibrosis) appears likely since mutual interactions between vasoactive and structural peptides contribute to self-sustained structural/functional changes in vessel walls. Switching the large array of zinc metalloproteases on and off inside microcirculatory environments, particularly along the 7 m² nephron surface, remains an important task in renoprotective strategies intended to cope with the inherent risk of acute renal dysfunction, as observed at the beginning of generalised use of ACE inhibitors.

Structural nephron changes, even if heterogeneous during the involvement and progression of renal disease in diabetic patients, can no longer be considered as inevitable. Molecular and cellular mechanisms and interventions begin to be dissected out. New molecules interacting with proteolytic cascades along the ACE outcome are emerging and need to be precisely assessed by modern biochemical tools in basic, experimental, pre-clinical and clinical settings. Future assessment and intervention strategies are promising. MMP-transgenic mice, TIMP and TGFβ anti-sense and scrambled oligonucleotides, by intervening at the
transcription/translation level of fibrosis induction, could theoretically arrest degenerative disease progression and prolong native kidney duration either in place or in association with enzyme/natural substrate displacement by pseudomimetic substrates.

REFERENCES


