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Research Article

Endostatin Co-Localises with MMP9- in the Renal Tubular Cells in an Experimental Model Ischemia/Reperfusion

Milena C. Cichy[1], Katiucia B. S. Paiva[2], Maria L. Z. Dagli[3], Daniel S. Sanches[3], Nestor Schor[1], Maria H. Bellini[1,4*]

[1]*Nephrology Division, Federal University of Sao Paulo, 04023-900 São Paulo, SP, Brazil*

[2]*Department of Oral Pathology and Anatomy, Institute of Biomedical Sciences, University of São Paulo, 5508-000*

[3]*Department of Pathology, Veterinary Medicine School, University of São Paulo, 05508-270 São Paulo, SP, Brazil*

[4]*Biotechnology Department, Nuclear and Energy Research Institute, 05508-000 São Paulo, SP, Brazil*

**Corresponding author: Dr. Maria H. Bellini, Av. Professor Lineu Prestes 2242, Cidade Universitatia-Butatta-Sao Paulo/SP-Brasil, CEP-05508-000, Tel: 5511-31339607; E-mail: mhmarumo@terra.com.br*

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Abstract

We have previously shown the expression of endostatin (ES) and matrix metalloproteinase-9 (MMP-9) in kidney in response to acute kidney injury induced by ischemia/reperfusion. The purpose of this research was to investigate the effect of renal ischemia/reperfusion injury on the expression and localisation of ES and matrix metalloproteinase 9 (MMP-9). C57BL/6 male mice were subjected to 45 minutes of ischemia and subsequent reperfusion (24, 48, and 72 hours). Renal function was evaluated based on blood urea nitrogen and creatinine dosage. ES and MMP-9 expression were measured semi-quantitatively by western blot. Gelatinolytic activity was determined using *in situ* zymography (ISZ) and gelatin zymography (GZ). Localisation and co-localisation of ES and MMP-9 were evaluated using immunofluorescence. The ES protein levels observed during ischemia and after 72h of reperfusion were increased relative to those observed in the sham treatment group. Kidney MMP-9 protein levels increased during ischemia and at 48 and 72 hours of reperfusion. Local gelatinolytic activity as demonstrated by ISZ increased between the ischemic period and 48 h of reperfusion and decreased from 48 to 72 hours after reperfusion. The gelatinolytic activity quantification supported the ISZ data. The findings of this study indicate that MMP-9 could be involved in renal collagen XVIII cleavage and, consequently, in the release of ES. Therefore, these molecules could represent a new mechanism of cellular response in this kidney injury model.

Keywords: Acute Kidney Injury; Endostatin, MMP-9; Gelatinolytic Activity

Abbreviations

AKI` : Acute Renal Injury;

ATN : Acute Tubular Necrosis;

ROS : Reactive Oxygen Species;

MMP : Matrix Metalloproteinase;

NC1 : C-terminal Fragment;

ES : Endostatin;

ECM : Extracellular Matrix;

ISZ : *In situ* Zymography;

GZ : Gelatin Zymography;

GCF : Germ Cell Factor

Introduction

Acute renal injury (AKI) is defined as an abrupt reduction in kidney function, as reflected by increases in serum creatinine [1]. AKI is associated with increased morbidity, mortality, hospital stay duration, and healthcare costs [2]. Renal ischemia/reperfusion injury is a leading cause of AKI. A deficiency in blood supply (ischemia) can occur for a number of reasons, for example, hypotension linked to sepsis, the use of radiocontrast, or blood loss after surgery or trauma. The restriction of oxygen and nutrients and the accumulation of waste metabolites can cause acute tubular necrosis (ATN). The restoration of blood circulation (reperfusion) results in an inflammatory response and the consequent generation of reactive oxygen species (ROS), which cause protein oxidation, lipid peroxidation, DNA damage, and apoptosis [3]. Additionally, ROS play key roles in both matrix metalloproteinase (MMP) gene expression and the activation of pro-MMP (i.e., the latent form of MMP) [4].

Mammalian MMPs (24 members) comprise the most important family of secreted or membrane-anchored enzymes that act alone or in combination to cleave virtually all extracellular matrix (ECM) components and generate bioactive molecules. Several peri- and extracellular proteins are degraded or processed by MMPs [5]. Proteolytic targets of MMPs include substrates that modulate cell behaviour by altering cell signalling, leading to physiological and pathological processes such as differentiation, migration, proliferation, survival, apoptosis, and invasion [6-10]. MMPs are classified in six groups according their substrate specificity. Gelatinases (MMP-2 and MMP-9) are distinct members of the MMP family that cleave several basement membrane (BM) components, such as type III, IV, and VII collagen, fibrillin, laminin, entactin/nidogen, and dystroglycans [11]. MMP activity is highly regulated because the inappropriate degradation of the ECM would compromise tissue integrity [12] .

Type XVIII collagen is a heparan sulphate proteoglycan that is present in BMs throughout the body. Its C-terminal fragment (NC1 domain), endostatin (ES), has been found to inhibit angiogenesis and tumour growth by restricting endothelial proliferation and migration and by inducing endothelial cell apoptosis. ES is generated by proteolytic MMPs and other proteases, and it can remain associated with the BM (immobilised) or be released into circulation (soluble) [13]. Rehn and colleagues showed that the soluble and immobilised forms of ES have distinct biological activities [14]. Soluble ES regulates the apoptosis, proliferation, migration, and invasion of endothelial cells, whereas immobilised ES supports the survival and migration of endothelial cells [15]. Collagen XVIII was found in the BM zones of the Bowman's capsule and the proximal and distal tubules of the adult mouse kidney [16].

Our group observed ES and MMP-9 expression after acute kidney ischemia/reperfusion injury and purified ES-related protein from ischemic/reperfused kidneys [17,15]. These studies showed that soluble ES induces endothelial cell proliferation, whereas immobilised ES promoted endothelial cell adhesion, suggesting its role in endothelium survival.

In the present study, we used a murine acute kidney ischemic/reperfusion injury model (sham, ischemic, 24, 48, and 72 h after reperfusion) to evaluate the expression, localisation, and activity of ES and MMP-9.

Materials and Methods

Mice

C57BL mice (8–10 weeks old) were obtained from the animal facility at the Federal University of São Paulo, São Paulo, Brazil. All of the animals received care in accordance with the standards of the University under a protocol that was approved by the Animal Experimentation Ethics Committee (2008/217201732).

Acute Kidney Ischemia-Reperfusion Injury Model

Mice (n = 30) were anesthetised with an intraperitoneal (i.p.) injection of ketamine (125 mg/g body weight, Ketalar; Parke-Davis, USA) and xylazine (12.5 mg/g body weight; Phoenix Scientific, Inc., USA), after which a midline abdominal incision was made. Both renal pedicles were occluded for 45 min with a non-traumatic clamp. After the ischemia or reperfusion period, 0.5 ml of blood was collected from the inferior vena cava. The mice were sacrificed by cervical dislocation after ischemia, 24, 48 or 72h of reperfusion, and the kidneys were harvested. Sham animals (n = 6) underwent similar surgical procedures without renal pedicle clamping. The blood was centrifuged to obtain serum and stored at –20°C for biochemical analysis. One half of each kidney was embedded in Tissue-Tek® O.C.T. (4583 - Sakura), snap frozen in liquid nitrogen, and stored at –80°C for *in situ* zymography; the other half was immediately fixed in 10% neutral buffered formalin, embedded in paraffin, and routinely processed.

Renal Function

Urea (BUN) and plasma creatinine levels were determined us-

ing commercial kits (Urea UV Liquiform Cat. 104 and Creatinina K Cat. 96, respectively; Labtest Diagnostics, Brazil).

Protein Extraction and Western Blotting

The kidney tissue (100 mg wet wt) was homogenised in 2 ml of extraction buffer (0.1 M Tris, 0.01 M EDTA, 1% SDS, and 0.01 M DTT at pH 8.0). The homogenate was centrifuged at 3000 rpm for 10 min, and the supernatant was collected and stored at -20°C. The protein concentration was determined using the *DC* Protein Assay Kit I (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. SDS-polyacrylamide gel electrophoresis was conducted under reducing conditions. The 5% and 12% separating gels were overlaid with a stacking gel containing 4% polyacrylamide. Samples with the same protein concentration (50 µg/per lane) were separated at 100 V/plate for approximately 1.5 h, until the dye front reached the end of the gel. After electrophoresis, the proteins were electrotransferred onto nitrocellulose membranes at 50 V for 50 min. Following protein transfer, the blotted membranes were blocked with 5% non-fat dry milk for 1 h and incubated with primary antibodies against ES (AB1880, Millipore, Temecula, CA, USA, 1:200), pro-MMP-9 (ab38898, Abcam, Cambridge, UK, 1:200) and GAPDH (MAB374, Chemicon, Temecula, CA, USA, 1:500) overnight, and the blots were developed using goat anti-mouse IgG biotinylated secondary antibody (AP308P, Millipore, Temecula, CA, USA, 1:5000) for 1 h. The bands were visualised using enhanced chemiluminescence (ECL system; Amersham Bioscience, Piscataway, NJ, USA) according to the manufacturer's guidelines. The western blot signal was normalised to the GAPDH band density. The bands obtained by western blotting were quantified in an ImageJ software-based analysis [18].

Immunofluorescence

Immunofluorescence was performed at the Laboratory of Experimental Oncology, Department of Pathology, Faculty of Veterinary Medicine and Animal Science, University of São Paulo. Paraffin sections (3 µm in thickness) were mounted on SuperFrost Plus slides (Fisher Scientific, Pittsburgh, PA, USA), dried in an oven at 60°C, deparaffinised, and rehydrated. Heat-induced epitope retrieval was performed by immersing the slides in citrate buffer (0.1 ml/L, pH 6.0) and incubation in a microwave oven for 15 min. A block solution of hydrogen peroxide and methanol (1:4), milk 5% in PBS/Tween was used to block non-specific reactions, followed by incubation with primary antibody (the same antibodies used for western blotting) at a dilution of 1:100 overnight in a humidified chamber at 4°C. After washing, the slides were incubated for 2 h with an Alexa 488 A/M-green secondary antibody (Invitrogen Corporation, Eugene, OR, 1:100) to detect the primary antibody. After several washes with PBS, each section was counterstained using an antifade mounting media (ProLong Gold antifade reagent containing DAPI; Invitrogen Corporation, Eugene, OR). The fluorescence signals were observed under a fluorescence Nikon E-800 microscope (Tokyo, Japan).

***In situ* Zymography (ISZ)**

Gelatinolytic activity was evaluated in unfixed cryostat sections (8 µm thick) using DQ-gelatin as a substrate (FITC-conjugated, Enz-Chek; Molecular Probes, Eugene, OR). Cryosections of normal or injured kidney were air- dried for 10 min. DQ-gelatin (1:10 – 100 µg/mL) was diluted in reactive buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, 0.2 mM NaN_3, 0.5% low melting agarose, 0.03% Triton-X 100, pH 7.6), and this mixture (100 µl) was pipetted on top of the sections. The agarose was allowed to solidify at 4°C for 5 min, and then the sections were incubated at 37°C for 24 h. We used the general MMP inhibitor 1,10-phenanthroline (Phe) for the negative controls. Mouse ovary sections served as positive controls (Curry *et al*., 2001). Finally, sections were fixed in 4% formaldehyde, washed in PBS, and mounted in antifade media with DAPI (Vectashield, Vector Laboratories). Sections were analysed using a Leica fluorescence microscope.

Gelatin Zymography (GZ)

Supernatants of 50 µg of total protein from kidney homogenate from each animal were used for gelatin zymography. Briefly, electrophoresis was carried out on 10% polyacrylamide gels containing 0.1% gelatin type A (Sigma, St. Louis, USA) under nonreducing conditions. After electrophoresis, the gels were washed in 2.5% Triton X-100 to remove sodium dodecyl sulphate, incubated in reaction buffer (0.05 M Tris-HCl pH 8.8; 5 mM $CaCl_2$; 0.02% NaN_3) for 16 h at 37°C, and stained with 0.1% Coomassie Brilliant Blue R250 (Sigma). Densitometric analysis of the gels was performed using image software. MMP-9 concentration was estimated based on its gelatinolytic activity.

Statistical Analysis

The results are presented as the means ± SE. Single comparisons of mean values were performed using Student's t test. Multiple comparisons of mean values were performed using one-way ANOVA followed by Bonferroni's test, using GraphPad Prism version 4.0 for Windows [19]. $P < 0.05$ was considered statistically significant.

Results

Renal function after ischemia/reperfusion

In this work, we used a model of nonlethal kidney injury. The creatinine and BUN plasma levels of ischemic and reperfused animal groups were compared with those of sham-operated mice. Marked deterioration of renal function, i.e., the highest creatinine and BUN plasma levels, were observed 24 h after

reperfusion (Figure 1). The mice exhibited a pronounced and statistically significant decrease in creatinine and BUN serum at 48 h post-ischemia.

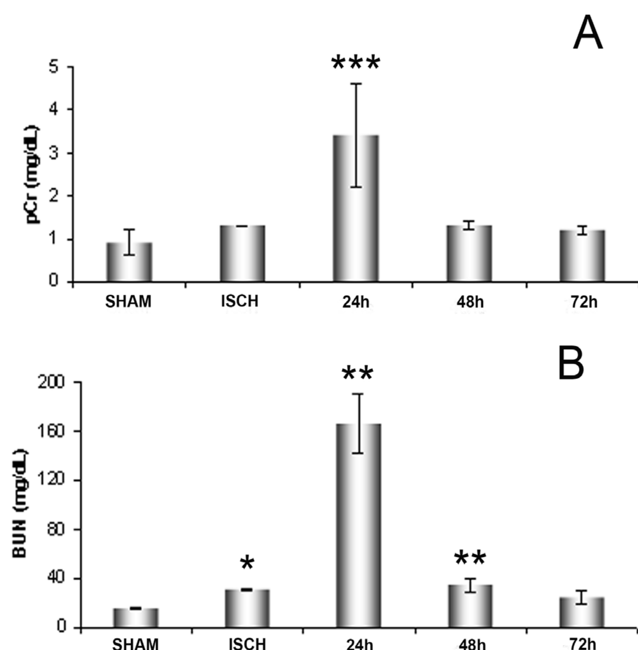

Figure 1. Biochemical parameters of renal function after ischemia/reperfusion injury. (A) Plasma creatinine (pCr) and (B) blood urea nitrogen (BUN) levels. One-way ANOVA (* P<0.05, ** P<0.01, ***P<0.001).

The ES and MMP-9 protein levels were measured in each of the experimental groups. Western blot analysis of the total kidney protein revealed a 28 kDa ES isoform and demonstrated that ES expression was upregulated during ischemia (sham *vs* ischemia - P<0.05) and after 72h of reperfusion (sham *vs* 72 hours of reperfusion - P<0.05) (Figure 2A and 2B). Only the latent form of MMP-9 could be detected in the kidney tissue using western blot (Figure 2A). Latent MMP-9 expression were increased in ischemic mice compared with sham-treated mice (*P*<0.05), decreased from the ischemic period to 24 hours after reperfusion, and finally exhibited a progressive increase from 24 to 72 hours after reperfusion (sham *vs* 48 h, *P*<0.01; sham *vs* 72 hours *P*<0.001) (Figure 2A and 2B). No significant difference between latent MMP-9 and ES relative protein levels was observed in the sham, ischemic and 24 hours after reperfusion (Figure 2C). In contrast, latent MMP-9 expression was increased relative to ES expression at 48 and 72 hours (2-fold and 1-fold, respectively) (Figure 2B, 3).

Both MMP-9 and ES expression were detected in the glomerulus and in the tubuli in all experimental groups. Nuclear and diffuse cytoplasmic immunostaining were observed for MMP-9 and ES, respectively. We observed increased ES and MMP-9 expression in the ischemic group compared with the sham group, decreased expression from the ischemic period to 24 h after reperfusion, and progressively increased expression from 24 to 72 hours after reperfusion (Figure 3). This pattern was similar to that observed in the protein levels. Furthermore, MMP-9 and ES were co-localised in the tubule nuclei and cytoplasm (Figure 3).

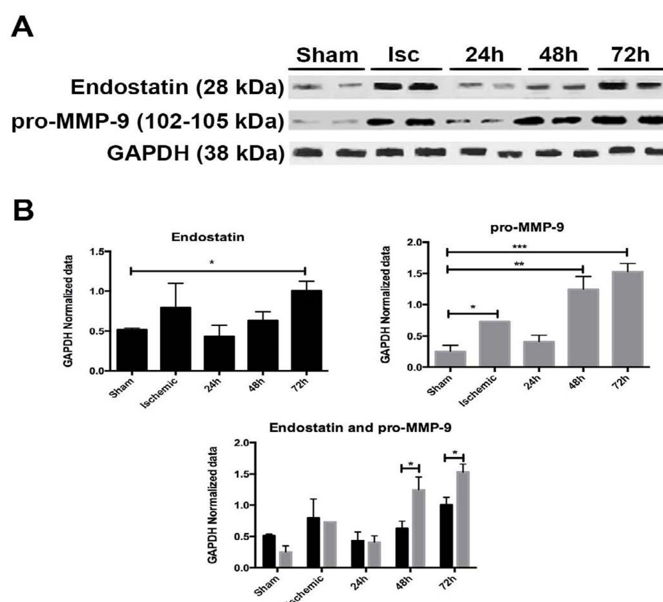

Figure 2. Quantification of relative MMP-9 and ES protein levels after ischemia/reperfusion injury. (A) SDS-PAGE gel and (B) relative densitometric quantification. One-way ANOVA (**P*<0.05, ***P*< 0.01, ****P*<0.001).

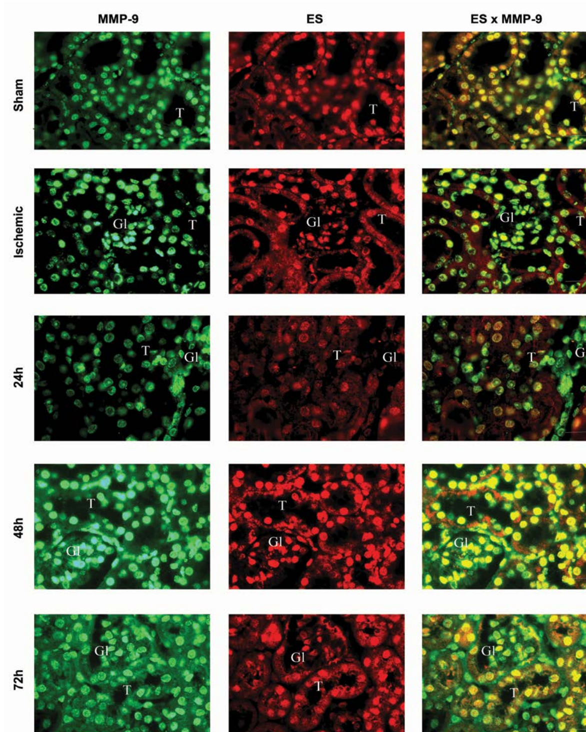

Figure 3. Immunolocalisation of MMP-9 and ES after ischemia/reperfusion injury. MMP-9-FITC (A, D, G, J and M), ES-rhodamine (B, E, H, K and N), and merge (C, F, I, L and O). Gl: glomerulus; T: tubule. Scale bar: 20 µm.

The enzymatic activity of MMP-9 was assessed using two different methods. First, the localisation of the gelatinolytic activity was determined by ISZ; green fluorescence corresponds to gelatin-FITC degradation by gelatinolytic enzymes in frozen sections. We observed green areas in the glomerulus as well as in the tubuli in all experimental groups. The fluorescence intensity progressively increased from the ischemic period to 48 hours after reperfusion and decreased from 48 to 72 hours after reperfusion (Figure 4A). Second, gelatinolytic activity was quantification by GZ from gelatin-coated polyacrylamide gels. We detected an increase in active MMP-9 (86 kDa) in the ischemia group compared with the sham group, a decrease from the ischemic period to 24h after reperfusion, an increase from 24 to 48 and 72 hours after reperfusion (sham *vs* 48 hours $P<0.05$; sham vs 72 hours $P<0.05$ ((Figure 4B and 4C). The maximal MMP-9 enzymatic activity at 48 hours after reperfusion was confirmed by relative densitometric quantification of the active form of MMP-9 (Figure 4C).

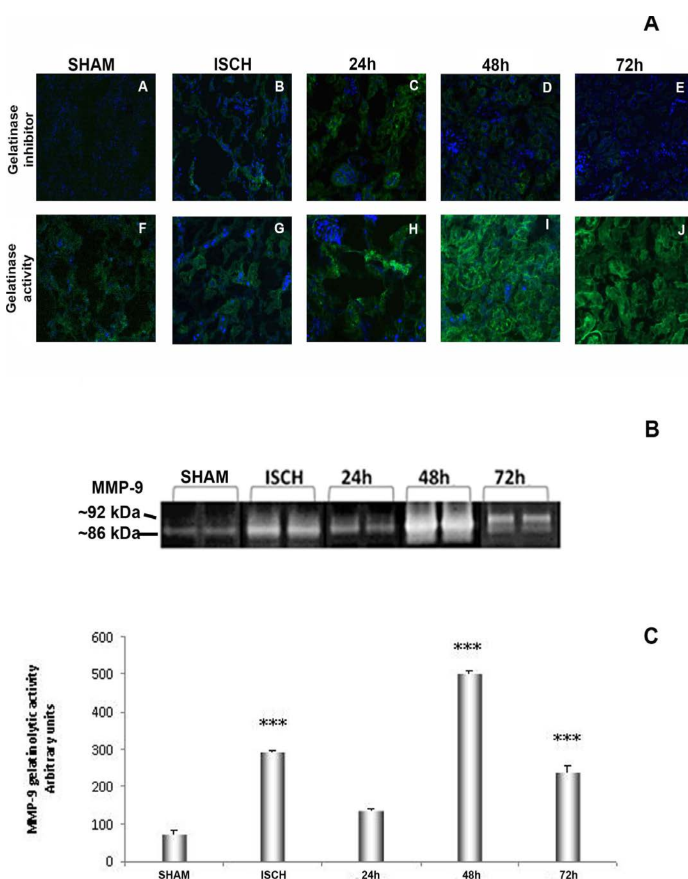

Figure 4. Localisation and relative quantification of MMP-9 enzymatic activity. (A) ISZ, (B) GZ and (C) relative densitometric quantification of active MMP-9 band. One-way ANOVA (***$P<0.001$).

Discussion

MMPs are expressed in the normal kidney (MMP-2, -3, -9, -13, -14, -24, -25, -27, and -28); however, the distribution of these enzymes is complex and not fully characterised. AKI induces the expression of the same MMPs (MMP-2, -7, -9 and -14) that participate in disease progression after ischemia/reperfusion injury. These MMPs promote cell death, loss of cell-cell adhesion, and endothelial permeability [20,21]. In vivo functional studies have employed MMP-2 and MMP-9 knockout mice and examined the effects of MMP inhibition using synthetic inhibitors [22-28]. The pathological processes involved in the increase in MMP activity following ischemia/reperfusion injury are inflammation and oxidative or nitrosative stress. However, the extent and duration of MMP activity are modulated by the degree of ischemia/reperfusion-induced injury. In this way, MMPs may be associated with both tissue damage and tissue repair [29].

Extracellular matrix (ECM) components are important during morphogenesis as well as in preserving tissue integrity. Ischemic injury promotes a series of events including the remodelling of the ECM. Changes in the ECM can be observed within minutes after renal injury [30]. Our group localised collagen XVIII in the kidneys and showed that it is cleaved during ischemic/reperfusion injury, generating ES; however, the mechanism of this collagen breakdown is unclear [17]. It is known that MMPs (MMP-3, -7, -9, -13, -14, and -20) can proteolytically convert collagen XVIII into biologically active endostatin [31-35].

Here, we found that MMP-9 expression (western blot data) and gelatinase activity (zymogram data) were modulated. Active MMP-9 could not be detected by western blot but could be detected with the more sensitive zymogram assay.

MMP-9 was expressed mainly in the proximal tubules and collectors, and the expression of its active form was increased at all-time points during ischemia and reperfusion, with peaks during ischemia and at 48 hours of reperfusion. Our analysis showed a positive correlation between the expression of ES and the gelatinolytic activity of MMP-9, indicating that, in this microenvironment, MMP-9 may be one of the molecules that are responsible for the cleavage of collagen XVIII. Studies of knockout mice indicated that MMP-9 is an important regulator of renal function [24]. According to these authors, germ cell factor (GCF), a membrane-bound protein of 45kDa, is a substrate of MMP-9. MMP-9 cleaves GCF, promoting the release of the soluble form, which then plays a role in blocking tubular cell apoptosis. Moreover, Caron et al suggest that another in vivo substrate of MMP-9, ZO-1 (a component of the zonula occludens), was degraded by MMP-9 during ischemia, showing that the increase in MMP-9 during ischemia is functional [36]. It was also reported that an increase in the activity of the MMP-9 monomer due to the decrease of TIMP-1 during reperfusion

may contribute to glomerular recovery.

Immunofluorescence localisation and co-localization analysis showed that MMP-9 localizes primarily to the nucleus but is also present in the cytoplasm and extracellular space at 48 and 72 h after reperfusion, when the tissue regeneration process is evident. The ES showed preferential localisation to the basement membrane and to cytoplasmic tubule cells. In this micro-environment, at 48 and 72 h of reperfusion, there was a clear co-localization of these two molecules. This result suggests that MMP-9 acts in the cleavage and subsequent release of ES in the renal tissue. Collagen XVIII is a substrate of MMP-9, and its cleavage generates fragments (the ES isoforms) that, in soluble form, have antiproliferative activity. In recent work, Bellini et al and Wickstrom et al demonstrated that the immobilised ES promotes cell adhesion and therefore may assist in the recovery of endothelial cells [15,37].

In conclusion, ES protein expression is increased in renal injury and recovery. The peak expression of ES coincides with periods of greater MMP-9 gelatinase activity. The immunofluorescence studies confirmed the co-localization of ES and MMP-9, indicating that collagen XVIII / ES may be a substrate of MMP-9. Our results also suggest that these two molecules have important roles in the regeneration of renal tissue.

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