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Original Article

Dysregulated matrix metalloproteinases and tissue inhibitors of metalloproteinase in residual splenic tissue after subtotal splenectomy due to portal hypertension

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Abstract: Objective: This study was designed to measure the mRNA and protein levels of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinase (TIMPs) in residual splenic tissue and serum after subtotal splenectomy due to portal hypertension, to investigate splenic fibrosis in molecular detail and the value of reserving splenic tissue during surgery. Methods Thirteen cases of splenomegaly due to portal hypertension that were removed surgically were examined in splenomegaly group. After eight years, another specimen was obtained for the residual spleen group by puncture biopsy. Additionally, 13 traumatic spleen samples were collected for the control group. Immunohistochemistry, enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) was used to detect MMP and TIMP mRNA and protein levels in residual spleen samples and serum. Results in the residual spleen, splenomegaly and control groups, MMP-2, MMP-9, TIMP-1 and TIMP-2 expression were densely distributed in macrophages. A significantly higher ratio of cells were positive for MMP-2, MMP-9, TIMP-1 and TIMP-2 protein and mRNA expression in tissue from the residual spleen and splenomegaly groups compared with the control group ($P<0.01$ and $P<0.05$, respectively); moreover, there were no significant differences between tissue from the residual spleen and splenomegaly groups ($P>0.05$). Significantly higher levels of serum MMP-2, MMP-9, TIMP-1 and TIMP-2 were observed in the residual spleen and the splenomegaly groups compared with the control group ($P<0.01$); but there were no significant differences between the residual spleen and the splenomegaly groups in serum levels of these markers ($P>0.05$). Conclusion After subtotal splenectomy, MMP-2, MMP-9, TIMP-1 and TIMP-2 expression were increased in residual splenic tissue and serum in patients with portal hypertension. The results suggested that dysregulated MMPs and TIMPs expression occurs in residual spleen after subtotal splenectomy due to portal hypertension.

Keywords: Residual spleen, portal hypertension, splenomegaly, matrix metalloproteinases, tissue inhibitors of metalloproteinase

Introduction

The spleen is an important immune organ, and splenomegaly is one of the complications of portal hypertension that results from a continuously high hemodynamic state, which gradually leads to splenic tissue remodeling, including hyperplasia of vessels and lymphoid tissues and fibrosis [1-3]. These complications lead to difficult choices during surgery regarding how to treat portal hypertension-associated splenomegaly. The contending factors for performing splenic-preserving surgery versus total splenectomy include the risks of portal thrombosis and/or negatively affecting splenic immune functions and whether surgery can correct hypersplenism and/or decrease portal hypertension [4, 5]. For example, previous research has shown that splenomegaly can result in the abnormal metabolism of extracellular matrix in splenic tissue, which may result from a dysregulation in the balance between matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinase (TIMPs) [6, 7].

MMPs are a family of enzymes that act on all extracellular matrices through a common substrate. Traditionally, MMPs were only consid-

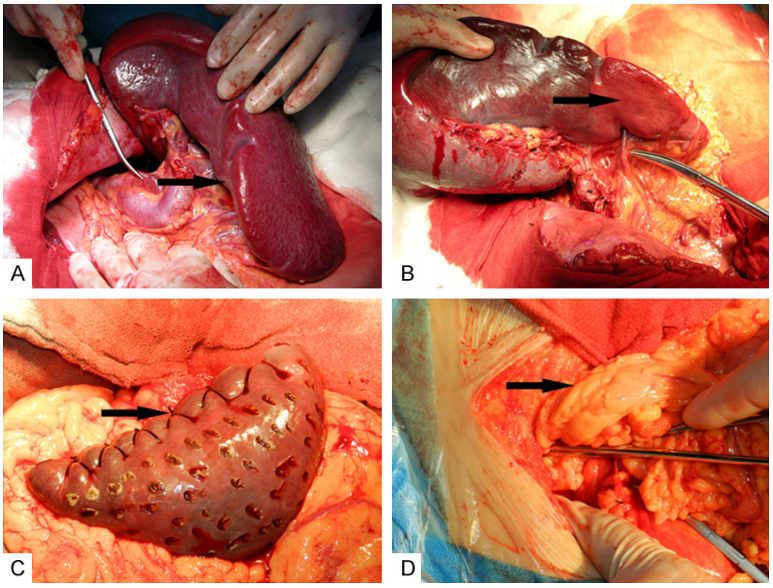

Figure 1. Operative method. A: The lienorenal and splenophrenic ligaments were ligated, and the spleen was moved through the incision. B: The left gastroepiploic and splenocolic vessels were preserved, and the spleen was presented the ischemic separatrix. C: The residual splenic section was sutured using a horizontal mattress cross-suture method, and the splenic capsule was cauterized with an electrotome to create a rough surface. D: The omentum majus (10×5 cm) was excised, and was tamponaded into the retrosternal space and fixed in place (the arrow indicates).

ered to participate in the degradation and renewal of extracellular matrix; however, recent studies using genetic knockout animal models have shown that proteomics can alter the function of MMPs, and that individual MMPs have overlapping functions (excess and compensatory mechanisms), ambiguous function (protection or destruction of extracellular matrix), novel substrates and tissue specific expression, and involvement in previously unappreciated biological processes (proliferation and migration) [8]. It is now appreciated that the key functions of MMPs are to regulate a diverse set of cell characteristics and signaling pathways [8]. MMPs are secreted by connective tissue and proinflammatory cells, including fibroblasts, osteoblasts, endothelial cells, macrophages, neutrophils and lymphocytes. There are now 30 members of the MMP family, and MMP-2 and MMP-9 belong to the gelatinase sub-family [9, 10]. TIMPs are multifunctional proteases that regulate proliferation, apoptosis, MMP activity and vascular proliferation. TIMPs function primarily through the mitochondrial pathway of MMPs [11]. TIMPs are secreted by macrophages and connective tissue cells, and are expressed by endothelial cells and fibroblasts around blood vessels in tissues [12].

We hypothesized that there may be changes in MMPs and TIMPs in residual splenic tissue and serum after subtotal splenectomy due to portal hypertension; currently, this has not been investigated in the literature. In this study, we detected MMP-2, MMP-9, TIMP-1 and TIMP-2 expression in splenic tissue and serum by immunohistochemistry, enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR), and investigated the molecular mechanisms underlying splenic fibrosis and the clinical value of spleen-preserving surgery.

Materials and methods

Ethical approval of the study protocol (No. 1678) was obtained from the Human Research Ethics Committee of the 89th Hospital of the People's Liberation Army (Weifang, China). All individuals provided written informed consent.

Clinical data

Our studies relating to subtotal splenectomy began in 1984. As of 2015, we have conducted 852 subtotal splenectomies (preserving the lower pole, normal size of splenic tissue, splenic omentum, and splenocolic vessels for the purpose of blood supply). There were 167 cases of splenic trauma and 685 cases of splenomegaly due to portal hypertension (**Figure 1A-D**). Thirteen tissue samples were collected from patients in the 89th Hospital of the People's Liberation Army. Thirteen patients (seven males and six females; mean age, 31 years; age range, 26-36 years) had splenomegaly and cirrhosis and had undergone subtotal splenectomies plus fixation of the posterior sternal omentum majus. Patients were confirmed to have cirrhosis after hepatitis B infection; hepatitis B virus DNA was negative and patients were classified as class A or B according to the Child-Pugh classification. Cirrhosis was accompanied by hypersplenism with light or moderate varicose veins of the lower esophagus, and the fibrosis level in the spleen was III. Color

Table 1. Primer sequences used for reverse transcription polymerase chain reaction analysis of MMP-2, MMP-9, TIMP-1, TIMP-2

Gene	Primer	Amplified fragment length
MMP-2	Upstream: 5'-ATCTTTGCTGGAGACAAATTCTGGA-3'	175 bp
	Downstream: 5'-GCTTCAGGTAATAGGCACCCTTGA-3'	
MMP-9	Upstream: 5'-ACGCACGACGTCTTCCAGTA-3'	94 bp
	Downstream: 5'-CCACCTGGTTCAACTCACTCC-3'	
TIMP-1	Upstream: 5'-CCTTATACCAGCGTTATGAGATCAA-3'	194 bp
	Downstream: 5'-AGTGATGTGCAAGAGTCCATCC-3'	
TIMP-2	Upstream: 5'-CTGGACGTTGGAGGAAAGAAGG-3'	148 bp
	Downstream: 5'-CCATCTGGTACCTGTGGTTCAGG-3'	
GAPDH	Upstream: 5'-GCACCGTCAAGGCTGAGAAC-3'	138 bp
	Downstream: 5'-TGGTGAAGACGCCAGTGGA-3'	

Doppler ultrasound was used before surgery and 8 years after surgery to measure the size of the spleen. The pre-operative long diameter was 48 ± 5 cm, the transverse diameter was 30 ± 4 cm, and the pachy-diameter was 10 ± 2 cm. The post-operative long diameter was 11 ± 1 cm, the transverse diameter was 7 ± 1 cm, and the pachy-diameter was 4 ± 1 cm. Spleen organization was obtained by operating on the splenomegaly group. Patients who underwent splenic puncture to check for spleen organization 8 years after surgery formed the residual spleen group. Guided by color Doppler ultrasound, a hollow-needle biopsy was used to obtain samples of residual splenic tissue. Thirteen patients (seven males and six females; mean age, 30 years; age range, 28-37 years) who underwent splenectomies, but who did not have splenomegaly, comprised the control group.

Specimen collection and processing

Specimens were fixed in 10% formalin, dehydrated, embedded, and serially sliced into 4 μm sections. One slice of each sample was used for hematoxylin and eosin staining (H&E kit; Labest Biotech Co. Ltd, Beijing, China). Each specimen was cut into 15 slices, with five slices per group. In addition, the fresh specimens were collected and immediately inserted in EP tubes, then were preserved by Liquid Nitrogen and made PCR testing.

Immunohistochemistry staining

Immunohistochemistry was performed as follows: antigen retrieval was performed using ethylenediaminetetraacetic acid (pH=8.5), 0.3% hydrogen peroxide, and goat serum to blockthe non-specific antigen. The immunohistochemical SP method was performed. Sections were then incubated overnight at 4°C with primary antibody (rabbit anti-human MMP-2, MMP-9, TIMP-1, and TIMP-2 monoclonal antibodies were used to label the corresponding antigens), followed by incubation at room temperature for 30 min with biotinylated secondary antibody with diaminobenzidine as the substrate, and stained with hematoxylin. The negative control group was studied using the same steps described above, but primary antibody was used instead of phosphate-buffered saline.

Measurement of MMP-2, MMP-9, TIMP-1, and TIMP-2 levels in serum by ELISA method: Peripheral venous blood samples were taken 3 ml in fasting, stored at 4°C overnight, and centrifuged (2000 g/min for 10 minutes). Then, the supernatant was decanted into a sterile vial and kept at 80°C. All of the processes were performed strictly according to the instructions provided by the manufacturers.

Measurement of MMP-2, MMP-9, TIMP-1, and TIMP-2 mRNA expression by fluorescence quantitative PCR: splenic tissues and total RNA were extracted in each group and according to the instructions provided by Trizol (Invitrogen company), then was dissolved in DEPC treated demineralized water and kept at 80°C Fluorescence Quantitative PCR was used SYBR Green method. Related primer sequences (**Table 1**), housekeeping gene GAPDH as an internal calibration in experiment, ultrapure water (RNase-free) as a negative control was used. Analysis results were calculated by $2^{-\Delta\Delta Ct}$ amplification efficiency in each group.

Reagents and equipments

The immunohistochemical expression of MMP-2, MMP-9, TIMP-1, and TIMP-2 markers were purchased from Zhongshan Golden Bridge Co. Ltd (Beijing, China). SYBR PrimeScript RT-PCR Kit II as well as designed and synthesized primer sequence was purchased from TaKaRa Co.

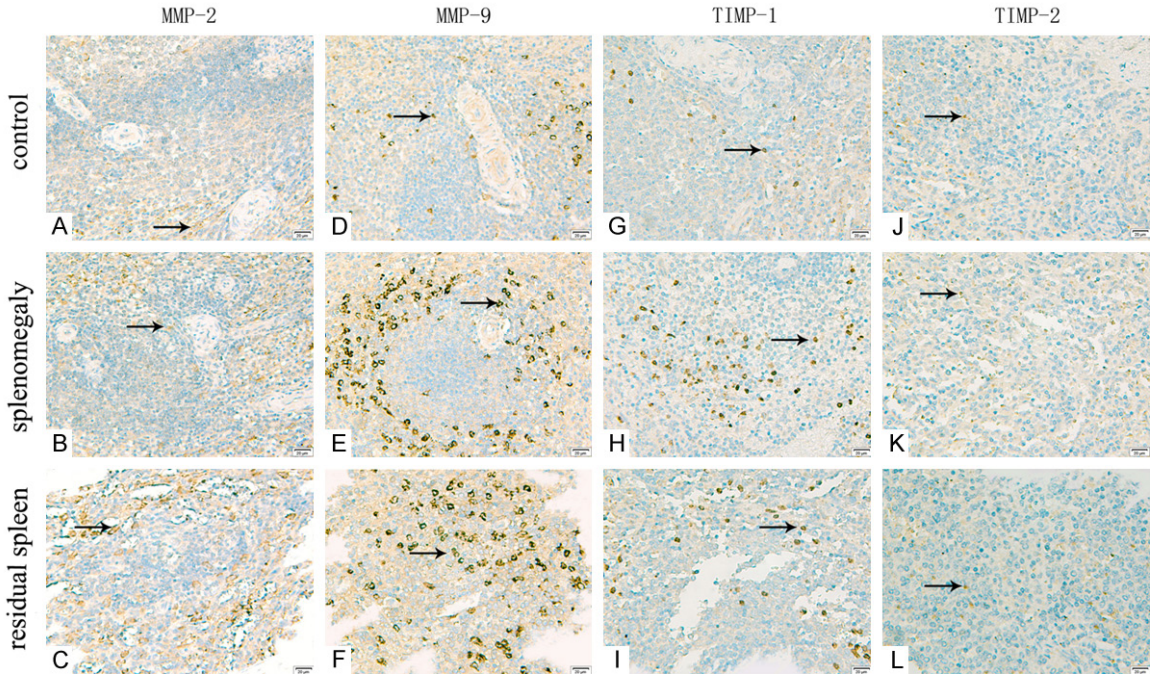

Figure 2. The expression of MMP-2, MMP-9, TIMP-1, and TIMP-2 proteins in the three splenic tissue. The expression of MMP-2 protein in the control, the splenomegaly, and the residual spleen (A-C). The expression of MMP-9 protein in the control, the splenomegaly, and the residual spleen (D-F). The expression of TIMP-I protein in the control, the splenomegaly, and the residual spleen (G-I). The expression of TIMP-2 protein in the control, the splenomegaly, and the residual spleen (J-L). (the arrow indicates positive expression, Immunohistochemistry, ×400 magnification, Bars, 20 μm).

Ltd (Dalian, China); MMP-2, MMP-9, TIMP-1, and TIMP-2 ELISA kits were purchased from Jing Mei Co. Ltd (Shenzhen, China). enzyme-labeled instrument (Versa Max type; Molecular Devices, Sunnyvale, USA). Fluorescence quantitative PCR (Bio-RadIQ5 type; Bio Rad, CA, USA) Light microscope (BX51 type; Olympus, Tokyo, Japan).

Criterion standard

The cells in which the cytoplasm had brown-yellow or dark brown granules were referred to as positive cells; no staining or light yellow was referred to as negative cells. For the histologic analysis, five fields per section were examined for staining at 400× magnification. The immunohistochemical expression of MMP-2, MMP-9, TIMP-1, and TIMP-2 immunoreactive proteins were determined according to the intensity of nuclear staining and the density and morphology of chromatin. The results were evaluated by two independent investigators who were blinded to the patients' clinical findings. Based on MMP-2, MMP-9, TIMP-1, and TIMP-2 immunoreactive proteins were considered positive for the proteins ratio [number of positive cells/number of total cells (per high power field)] were counted. Under light microscopy (×400), Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA) was used to calculate the positive expression ratio of apoptotic cells and the apoptotic proteins, respectively. Two-to-five sections per specimen were evaluated.

Statistical analysis

SPSS (version 17.0; SPSS, Inc., Chicago, IL, USA) was used. Data are expressed as the mean ± standard deviation. Differences in measurement data were compared using one-way ANOVA, Student's t-test with two independent samples and two paired samples. A $P<0.05$ was considered significant.

Results

Distribution of MMP-2, MMP-9, TIMP-1, and TIMP-2 proteins expression in spleens

In marginal zone, positive expression of MMP-2, MMP-9, TIMP-1, and TIMP-2 proteins were densely distributed in the macrophage; In the red pulp, Scattered MMP-2, MMP-9, TIMP-1,

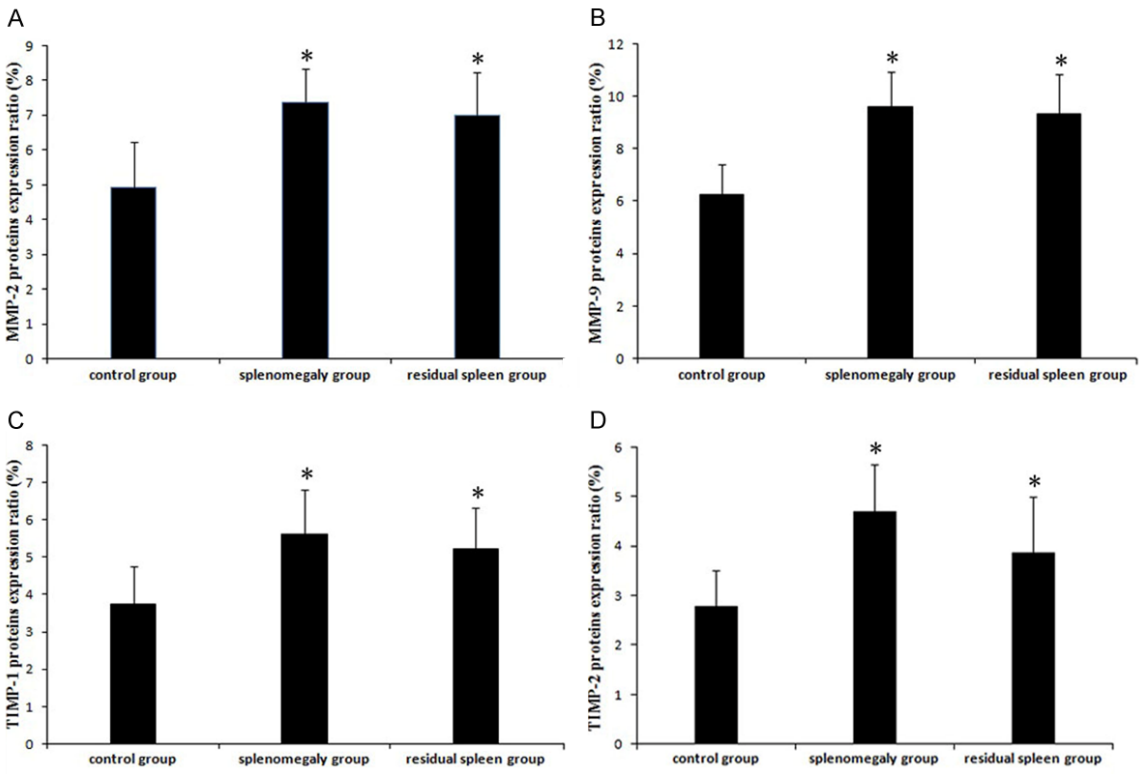

Figure 3. The positive expression ratio of MMP-2, MMP-9, TIMP-1, and TIMP-2 proteins in the control, the splenomegaly and the residual spleen groups (the cellular percentage/Per High-Power Field). *P<0.01 vs. control group.

and TIMP-2 proteins were present in endothelial cells of splenic capsule, fibroblast cells and reticular cells in splenic cord and spleen trabecula as well as the vessels around fibroblast cells; In the white pulp, the positive protein expression was rare. The distributions of MMP-2, MMP-9, TIMP-1, and TIMP-2 proteins positive expression were similar among the three groups (**Figure 2A-L**).

Determination of MMP-2, MMP-9, TIMP-1, and TIMP-2 proteins positive expression ratio in spleens

MMP-2, MMP-9, TIMP-1, and TIMP-2 proteins positive expression ratio in the residual spleen and the splenomegaly groups were significantly higher than control group (all P<0.01), while there was no significant difference between the residual spleen and splenomegaly groups (P>0.05, **Figure 3A-D**).

Determination of MMP-2, MMP-9, TIMP-1, and TIMP-2 levels in serum

MMP-2, MMP-9, TIMP-1, and TIMP-2 levels in serum in the residual spleen and the splenomegaly groups were significantly higher than control group (all P<0.01), while there was no significant difference between the residual spleen and splenomegaly groups (P>0.05, **Figure 4A-D**).

Determination of MMP-2, MMP-9, TIMP-1, and TIMP-2 mRNA expression in spleens

MMP-2, MMP-9, TIMP-1, and TIMP-2 mRNA expression in the residual spleen and the splenomegaly groups were significantly higher than control group (all P<0.05), while there was no significant difference between the residual spleen and splenomegaly groups (P>0.05, **Figure 5A-D**).

Discussion

In normal tissues, MMPs and TIMPs show low expression. When tissue remodeling is activated, their expression is rapidly induced, increasing their activity [13]. MMP activity is regulated by TIMPs, which bind in substrate- and tissue-specific manners to MMPs, blocking their proteolytic activity [14]. Studies have shown that at low concentrations, TIMP-2 promotes MMP-2

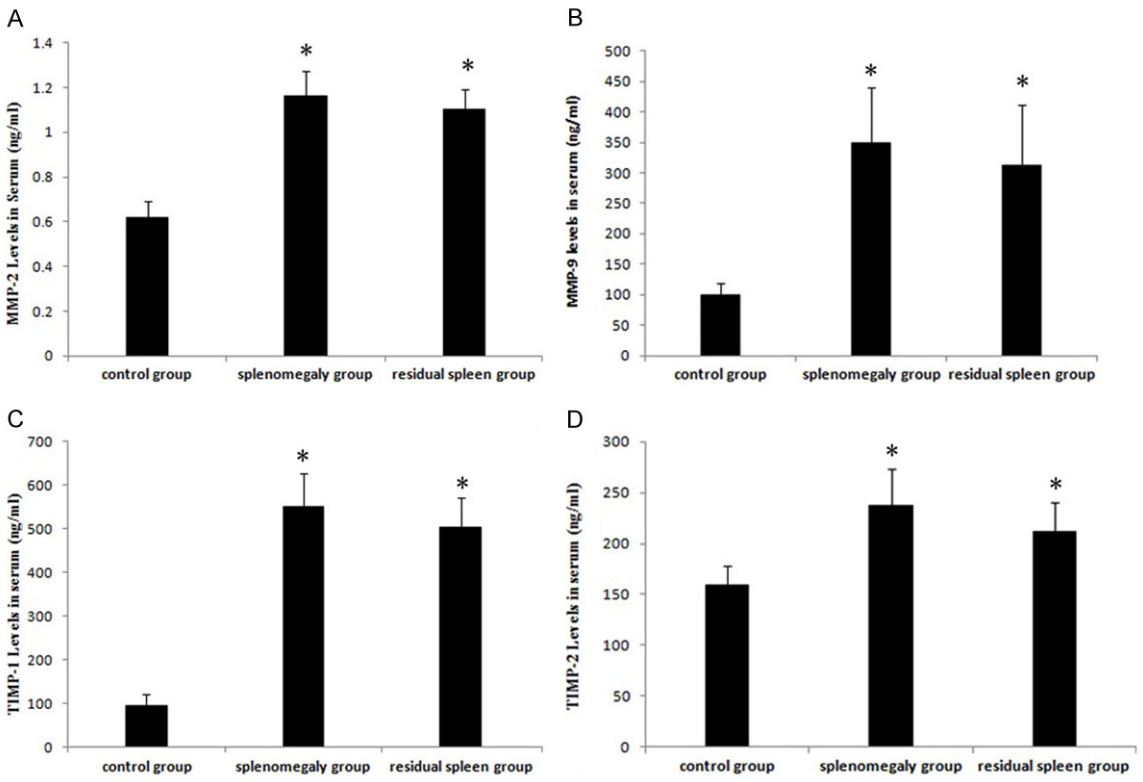

Figure 4. Serum levels of MMP-2, MMP-9, TIMP-1, and TIMP-2 in the control, the splenomegaly and the residual spleen groups (ng/ml). *P<0.01 vs. control group.

activity, while at high concentrations; TIMP-2 inhibits MMP-2 activity [15]. MMP-2 and MMP-9 play an important role in preventing fibrosis by degrading collagen IV and V, while TIMPs downregulate MMPs activity, promoting fibrosis [16]. The cirrhosis rat model showed increased MMP-2 and MMP-9 protein expression, and decreased TIMP-1 and TIMP-2 mRNA expression. These results suggested that a reduction in the MMPs/TIMPs ratio promotes hepatic fibrosis formation following extracellular matrix metabolism [17, 18]. Therefore, MMPs can promote extracellular matrix degradation in pathologic conditions, even when regulated by endogenous TIMPs. Therefore, a dysregulated balance in TIMPs and MMPs is favorable for the development and progression of disease [19].

Our study showed that MMP-2, MMP-9, TIMP-1 and TIMP-2 protein expression in the residual spleen, splenomegaly and control groups were primarily distributed in macrophages. A significantly higher ratio of positive MMP-2, MMP-9, TIMP-1 and TIMP-2 protein and mRNA expression was observed in the residual spleen and splenomegaly groups compared with the control group. Moreover, there were no significant differences between the residual spleen and splenomegaly groups. Serum MMP-2, MMP-9, TIMP-1 and TIMP-2 proteins levels were higher in the residual spleen and splenomegaly groups than in the control group; there were no significant differences between the residual spleen and splenomegaly groups. In splenic tissues, our immunohistochemical results showed that MMP-2, MMP-9, TIMP-1 and TIMP-2 protein expression were in agreement with other studies [20]. The serum levels of MMPs and TIMPs proteins were the similar to the studies by Luo et al. [21] and Busk et al. [22]. The trend of MMP-2, MMP-9, TIMP-1 and TIMP-2 mRNA and protein expression also were similar. This study suggested that tissue fibrosis was increased in residual spleen and splenomegaly, and like hepatic fibrosis, can stimulate various cells, including macrophages, endothelial cells and fibroblasts. Tissue fibrosis also accelerated MMPs and TIMPs secretion, which increases the degradation of excess extracellular matrix. The high splenic intramedullary pressure that

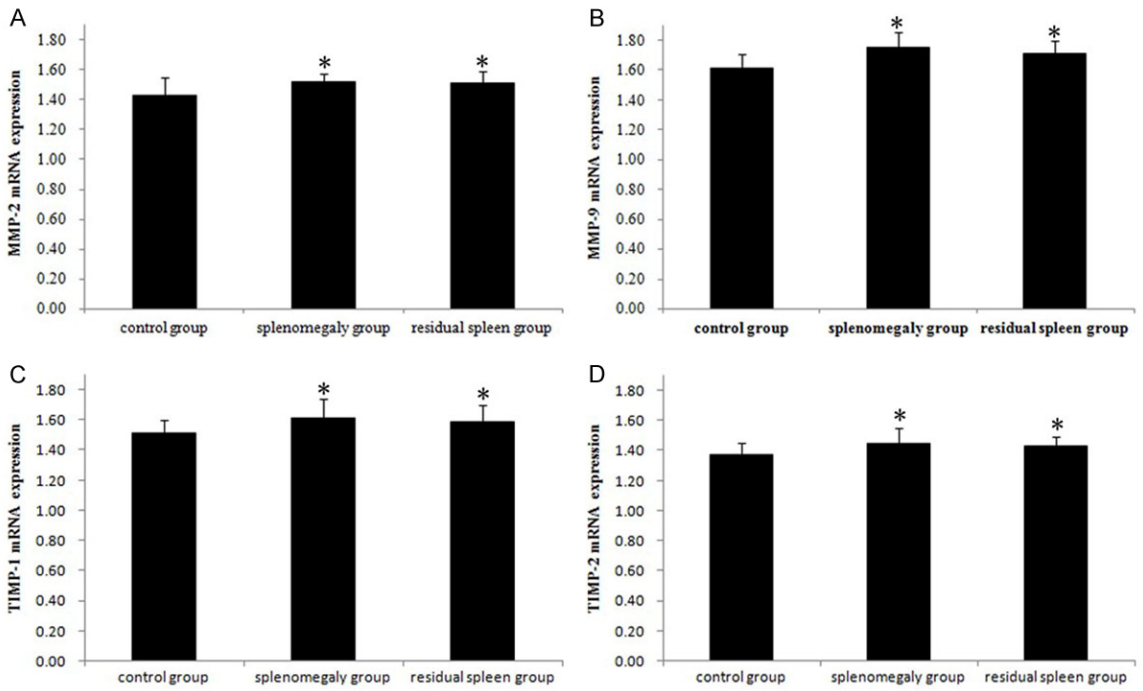

Figure 5. MMP-2, MMP-9, TIMP-1, and TIMP-2 mRNA expression in the control, the splenomegaly and the residual spleen groups. *P<0.05 vs. control group.

coexists with chronic inflammation in splenomegaly can be induced by increased MMP-9 and TIMP-1 mRNA expression [23]. It has also been confirmed that expression of MMP-2, MMP-9, TIMP-1 and TIMP-2, inflammatory cells and the release of inflammatory factors were closely related [24]. In normal conditions, MMP activity is low in tissues, but when inflammatory factor expression, growth hormone and/or oxygen stress were increased, MMP activity is induced and degrades extracellular matrix [25]. The degradation of extracellular matrix can affect apoptosis, and disturbances in the MMPs/TIMPs ratio may change tissue function and cell fate [26]. Our previous study showed that apoptosis was decreased in splenomegaly, while apoptosis was increased in residual spleen tissues [27]. These results suggested that there is no connection between the activity of MMPs and TIMPs in splenic tissue and cell renewal. Gulino et al. [28] proposed that hypoxia may lead to increased secretion of MMP-9 and TIMP-1, and cause severe disturbances in the MMP-9/TIMP-1 ratio. Gunia et al. [29] found that MMP-1 and TIMP-1 were imbalanced in septicopyemia spleens, and that the increase in MMP-1 was related to the activation of macrophages. It has become accepted that tissue fibrosis reflects disturbances in extracellular matrix synthesis and degradation, which are triggered by many factors, such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), platelet-derived growth factor (PDGF), MMPs and TIMPs [30]. The molecular pathways that regulate the balance of MMPs and TIMPs include p38/NF-κB, NF-κB, MAPK, PA-1, FAK and various other signaling pathways. Inducing and inhibiting signals for MMPs are also found in the extracellular matrix itself (cell factors, growth factors, extracellular matrix MMP-induced factors, binding signals, extracellular matrix proteins, cell stress and morphological changes), which can activate or inhibit MMP expression via intracellular signal transduction [31-34]. Castillo-Briceno et al. [35] also showed that this dynamic physiological balance can be related to immune responses.

Our results were in agreement with our previous results of splenic histology (collagen and elastic fibers). Namely, after subtotal splenectomy there is no further development of fibrosis in the residual splenic tissue [36]. This study confirmed that the increase in MMP-2 and MMP-9 protein and mRNA expression in residual spleen tissues reflects the presence

of residual splenic fibrosis. The increases of TIMP-1 and TIMP-2 protein and mRNA expression are a compensatory event. We speculate that there is no further imbalance in the MMPs/TIMPs ratio in residual spleens. This study also suggested that real-time detection of serum MMP-1, MMP-2, TIMP-1 and TIMP-2 levels may be a sensitive biomarker for evaluating splenic fibrosis [37]. In conclusion, combining these data with the previously reported changes in various immune cells and clinical indexes, and the observation that splenic function was normal in the residual fibrotic splenic tissue, if there is no risk of hypersplenism recurrence and the residual spleen tissue can play a role in shunting blood and decreasing portal venous pressure [5, 38], our study suggested that there is an important clinical significance to splenic-preserving surgery for portal hypertension patients.

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Disclosure of conflict of interest

None.

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