Original Article

In vitro differences of apoptosis between residual spleens and splenomegaly due to portal hypertension

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Abstract: Objective: The etiology of splenomegaly remains elusive. The present study was designed to determine whether or not a difference in apoptosis exists between splenomegaly and residual spleen after subtotal splenectomy due to portal hypertension. Methods: Thirty-four cases of splenomegaly due to portal hypertension in which the spleens were removed surgically were examined as the splenomegaly group. After 8 years, other specimens were obtained by puncture biopsy, which comprised the residual spleen group. Immunohistochemical staining and electron microscopy were used to assess apoptosis. Results: A significantly higher apoptotic cells and Bax proteins in positive expression ratio as well as Bax, Caspase-3 mRNA expression was observed in the residual spleen and control groups compared with the splenomegaly group (all \( P < 0.05 \)), while the positive expression ratio of Bcl-2 proteins and Bcl-2 mRNA expression in the splenomegaly group was significantly higher than the residual spleen and control groups (\( P < 0.05 \)). Endothelial cells, lymphocytes, and macrophages had the same apoptotic morphologic characteristics in the three groups under the electron microscope. Conclusion: Residual spleen after subtotal splenectomy due to portal hypertension can lead to dysregulation of apoptosis via the mitochondrial pathway, which suggests apoptotic up-regulation in residual spleen.

Keywords: Residual spleen, apoptosis, splenomegaly, portal hypertension

Introduction

The spleen is thought to be central in regulating the immune system, a metabolic asset involved in endocrine function [1]. Overwhelming post-splenectomy infection leads to a mortality rate as high as 50% [2]. How to reduce the incidence of infections, maintain splenic immune function, or administer vaccinations are important issues for surgeons [3, 4]. Clinical practice shows that subtotal splenectomy applies to splenic injuries, splenomegaly for hematologic diseases, and portal hypertension [5-7]. There is still controversy regarding subtotal splenectomy as a treatment for splenomegaly due to portal hypertension in cirrhotic patients. Advocates for splenectomy believe that, with fibrosis and limited immune function in splenomegaly, recurrence of splenomegaly and hypersplenism may occur in residual spleen [8]. However, advocates inclined to preserve spleen argue that subtotal splenectomy may reduce portal venous pressure, correct hypersplenism, and retain immune function of the spleen [9]. Subtotal splenectomy includes two approaches, i.e., preserving the upper and lower poles of the spleen supplied by the gastrosplenic, spleno-omential, and spleno-colic vessels [9, 10].

In multicellular organisms, homeostasis is maintained by the balance between cell proliferation and cell death [11]. Apoptosis, or programmed cell death, is a physiologic form of cell death. The morphologic and biochemical features of apoptosis include cell shrinkage, chromatin condensation of dense buds out of the cells, cell division, phagocytic activity of the proximal cells, and DNA breakage of 200 base pairs [12]. Apoptosis is the key to maintain homeostasis and variation in multicellular organisms, and disorders of apoptosis are implicated in pathologic conditions [13]. Apoptosis can directly reflect the stability of the inner environment in splenic tissues [14].
Apoptosis between residual spleen and splenomegaly

Changes in the level of apoptosis in patients with splenomegaly and residual spleen after subtotal splenectomy have not been reported. The current study detected and analyzed the positive expression of apoptotic cells and apoptotic proteins, as well as apoptotic morphology in the spleen by terminal deoxynucleotidyl transferase-mediated nick-end labeling assay (TUNEL) staining, immunohistochemistry, and electron microscopy, and assessed whether or not a difference in apoptosis exists between splenomegaly and residual spleen after subtotal splenectomy due to portal hypertension in an effort to provide a theoretical basis for spleen-preserving surgery.

Patients and methods

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

Our studies relating to subtotal splenectomy (splenomegaly due to portal hypertension) began in 1999. As of 2016, we have conducted 296 cases subtotal splenectomies (preserving the lower pole, normal size of splenic tissue, splenic omentum, and splenocolic vessels for the purpose of blood supply, Figure 1A-D). One hundred-two tissue samples were collected from patients in the 89th Hospital of the People’s Liberation Army. Thirty-four patients (twenty-three males and eleven 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. Inclusion criteria: patients with 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 as well as cirrhosis was accompanied by hyper-splenism with light or moderate varicose veins of the lower esophagus. Color Doppler ultrasound was used before surgery and 8 years after surgery to measure the size of the spleen. The pre-operative long diameter was 49 ± 5 cm, the transverse diameter was 30 ± 4 cm, and the pachy-diameter was 10 ± 2 cm. The post-operative long diameter was 10 ± 1 cm, the transverse diameter was 8 ± 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. Thirty-four patients (twenty-three males and eleven 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 5 slices. TUNEL staining and immunohistochemical staining for these sections were performed.

One mm³ of fresh specimens was obtained from all of the groups. The specimens were fixed in 3% glutaraldehyde (Lebang Biotech Co. Ltd, Suzhou, China) for 24-48 h, dehydrated, embedded, and dried. The specimens were then made into 70 nm ultra-thin slices (UC7 ultrathin slicing machine; Leica, Germany). Slices were cleansed with water and soaked in a saturated aqueous solution of uranyl acetate (Zhenpu Biotech Co. Ltd, Shanghai, China). The slices were then cleaned with double-distilled water and soaked in lead citrate solution (Tianyuan Co. Ltd, Yingkou, China). The ultrastructural changes of venous structure cells were observed using a Hitachi H-7500 transmission electron microscope (Hitachi, Tokyo, Japan).

TUNEL staining

Apoptotic cells were detected with in situ labeling of TUNEL as follows: i. slice dewaxing--dehydrated in xylene for 1, 2 (overnight), and 3 h; ii. hydration--100%, 95%, and 80% for 2 min; iii. washed for 5 sec; iv. twice-washed with 1× phosphate-buffered saline for 5 sec; v. after wiping dry, dropwised Proteinase K (diluted in PBS 1:10), then incubated at 37°C for 10-20 min; vi. twice-washed in 1×PBS for 5 sec; vii. dehydrated in 90% ethanol for 5 sec in the cold; viii. dripped 20 µL of slice reaction liquid (18 µL of apop A and 2 µL of apop B using an anterior preparation), kept warm at 37°C in a wet box for 1-2 h; ix. thrice-washed with Buffer I for 5 min; x. HS sealing (diluted in Buffer I 1:100) at room temperature for 30-60 min; xi. SP-AP (diluted in Buffer I 1:500) at room temperature for 1 h (50 µL/slice); xii. twice-washed in Buffer II for 15 min; xiii. twice-washed in Buffer III for 1 min; and ixv. stained with NBT-BCIP. The cells in which the nucleus or cytoplasm had brown granules were referred to as apoptotic cells. Proteinase K, apop A, and apop B were provided by Roche Biotechnology Company (Basel, Switzerland).

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 block the non-specific antigen. The immunohistochemical SP method was performed. Sections were then incubated overnight at 4°C with pri-
mary antibody [rabbit anti-human Bcl-2 associated x proteins (Bax) and B cell lymphoma/leukemia-2 proteins (Bcl-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. The immunohistochemical expression of pro-apoptotic Bax, anti-apoptotic Bcl-2 markers was purchased from Zhongshan Golden Bridge Co. Ltd (Beijing, China).

Measurement of the expression in Bax, Bcl-2, and caspase-3 mRNA by fluorescence quantitative PCR

Splenic tissues and total RNA were extracted for each group according to the instructions provided by Trizol Invitrogen (Los Angeles, CA, USA), then dissolved in DEPC-treated demineralized water and kept at 80°C. Fluorescence quantitative PCR and the SYBR green method were used. Related primer sequences (Table 1), a housekeeping gene (GAPDH) as an internal calibration, and ultrapure water (RNase-free) as a negative control was used. The results were calculated based on 2^ΔΔCt amplification efficiency in each group.

Standards of staining evaluation

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 apoptotic cells was 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 TUNEL labeling and immunofluorescence in situ, Bax and Bcl-2 immunoreactive cells were considered positive for the apoptotic ratio [number of apoptotic 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 17.0.3, [2009]; SPSS, Inc., Chicago, IL, USA) was used. Data are expressed as the mean ± standard deviation. The positive expression ratio of apoptotic cells, apoptotic proteins, Bax, Bcl-2, and caspase-3 mRNA expression in the spleen were compared in three groups by One-Way ANOVA and SNK-q method, respectively. Study groups were compared with the control group using Student’s t test with two independent samples and two paired samples. A P < 0.05 was considered significant.

Results

Distribution of TUNEL staining-positive apoptotic cells in spleens

In the white pulp, positive apoptotic cells were densely distributed surrounding the central artery and its branches and peri-arterial lymphatic sheath. Scattered positive apoptotic cells were present in the marginal sinus and marginal zone, as well as the splenic capsule.
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In the splenic tissue, scattered positive apoptotic cells were distributed around the wall of the splenic sinusoids and small blood vessels. The distributions of positive apoptotic cells were similar among the three groups (Figure 2A-F).

Determination of TUNEL-positive cells ratio in spleens

The apoptotic ratio in the splenomegaly group was significantly lower than the residual spleen and control groups (all $P < 0.05$), while there was no significant difference between the residual spleen and control groups ($P > 0.05$). There was no significant difference between the red pulp, white pulp, and the marginal zone among three groups ($P > 0.05$, Table 2).

Distribution of the apoptotic proteins expression in spleens

In the residual spleen and control groups, considerably more cells with positive Bax expression were present, while there were only scattered cells with positive Bcl-2 expression. In the splenomegaly group, the results obtained with positive Bax and Bcl-2 expression were opposite compared with the residual spleen and control groups (Figure 3A-F).

Determination of the apoptotic proteins expression ratio in spleens

The positive expression ratio of Bax proteins in the residual spleen and control groups were significantly higher than the splenomegaly group ($P < 0.05$), while the positive expression ratio of Bcl-2 proteins in the splenomegaly group was significantly higher than the residual spleen and control groups ($P < 0.05$). There was no significant difference between the residual spleen and control groups ($P > 0.05$, Figure 4A, 4B).

Determination of Bax, Bcl-2, and caspase-3 mRNA expression in spleens

Bax and Caspase-3 mRNA expression in the residual spleen and control groups were signifi-
Apoptosis between residual spleen and splenomegaly

![Figure 3. The expression of apoptotic proteins in the three splenic tissue. The expression of apoptotic proteins in the control (A, D), the splenomegaly (B, E), and the residual spleen (C, F). (The arrow indicates positive expression, immunohistochemistry, ×400 magnification, Bars, 20 µm).](image)

![Figure 4. The positive expression ratio of Bax and Bcl-2 apoptotic proteins in the three groups (the cellular percentage/Per High-Power Field). A: Bax proteins expression, *P < 0.05 vs. splenomegaly group; B: Bcl-2 proteins expression, *P < 0.05 vs. The control and residual spleen groups.](image)

Spleen tissue samples from the control, residual spleen, and splenomegaly groups were analyzed for apoptotic proteins (Bax and Bcl-2). The expression levels were significantly higher in the splenomegaly group compared to the residual spleen and control groups (*P < 0.05). The residual spleen group did not show a significant difference compared to the control group (*P > 0.05).

**Ultrastructural changes of apoptotic cells in spleens**

In the residual spleen, control, and splenomegaly groups, the apoptotic morphologic features under the electron microscope were as follows: fuzzy mitochondrial cristae; crista breaking; medullary changes; rough endoplasmic reticulum thickening; degranulation; and margination of the nuclear chromatin were present in the endothelial cells (ECs), lymphocytes (LYMs), and macrophages (MØs) of the spleen. The normal morphologic features were as follows: normal mitochondria; rough endoplasmic reticulum; and nuclear chromatin (Figure 6A-F).

**Discussion**

In vertebrates, apoptosis includes endogenous and exogenous pathways. The endogenous
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Figure 5. The levels in Bax, Bcl-2, and Caspase-3 mRNA expression in the three groups. A: Bax mRNA expression, *P < 0.05 vs. splenomegaly group; B: Caspase-3 mRNA expression, *P < 0.05 vs. splenomegaly group; C: Bcl-2 mRNA expression, *P < 0.05 vs. the control and residual spleen groups.

Figure 6. Ultrastructural changes in the endothelial cells (ECs), lymphocyte (LYMs) and macrophages (MØs) in the three splenic tissue. A: ECs (uranium-lead double staining, ×40000 magnification, Bars, 700 nm). B: LYMs (uranium-lead double staining, ×20000 magnification, Bars, 1.0 µm). C: MØs (uranium-lead double staining, ×20000 magnification, Bars, 1.0 µm). D: ECs (uranium-lead double staining, ×40000 magnification, Bars, 700 nm). E: LYMs (uranium-lead double staining, ×20000 magnification, Bars, 1.0 µm). F: MØs (uranium-lead double staining, ×20000 magnification, Bars, 1.0 µm). M: Mitochondria; N: Nucleus; The arrow indicates rough endoplasmic reticulum.

pathways, also known as the mitochondrial pathway, regulates an apoptosis protein promoter (Bax or Bcl-2) and specific caspases (caspase-9) stimulate the release of cytochrome C from mitochondria into the cytoplasm combined with apoptotic protease activating
factor to trigger cell apoptosis. The exogenous pathway, also called the transmembrane channel, under apoptosis signal stimulation by FasL and tumor necrosis factor-alpha, affects corresponding receptors to induce cell apoptosis [15-17]. When the mitochondrial outer membrane permeabilizing occurred, cell death should not be avoided [18]. Normal mitochondrial transmembrane potential is necessary to maintain conditions for mitochondrial oxidative phosphorylation and produce triphosphaden triphosadenine. When the potential difference (mitochondrial exterior and interior) decreased, the mitochondrial transmembrane potential was reduced and the mitochondrial exterior and interior can undergo biochemical changes, such as cytochrome C and caspase activator releasing energy and apoptosis. Bcl-2 and caspase activation can produce cascade effect and stimulate apoptosis [19]. To control cellular integrity, apoptosis plays an important role, and is precisely regulated [20]. In the mitochondrial pathway, the dysregulated ratio of anti-apoptotic proteins (Bcl-2 and Bcl-xl) and the pro-apoptotic protein (Bax) determine the level of apoptosis (cell survival and death) [21]. Bax and Bcl-2 proteins are the main inducer and regulator of the mitochondrial apoptotic pathway, respectively. Bax apoptotic proteins can induce the release of cytochrome C from mitochondria into the cytoplasm; Bcl-2 apoptotic proteins can block Bax-inducing apoptosis by preventing the release of cytochrome C [22]. Under normal conditions, most Bax stays in the presence of monomers in the cytoplasm or adheres to the mitochondrial outer membrane. After activation, Bax combines with mitochondria as a dimer crosslinking membrane protein, and promotes apoptosis via the mitochondrial pathway. Bcl-2 is related to a different Bax, which is located on mitochondrial membrane proteins, combined with Bax forming heterodimers, to prevent apoptosis via the mitochondrial pathway [23]. When Bax plays a dominant role, it can accelerate apoptosis. Bcl-2 plays a dominant role, which is opposite to apoptosis [24].

Our study showed that the apoptotic cells of the spleen are densely distributed surrounding the central artery and its branches and periarterial lymphatic sheath, which is in agreement with the characteristics of the main splenic cells. The apoptotic ratio and mRNA expression of the splenomegaly was significantly lower than the residual and normal spleen, while the apoptotic ratios and mRNA expression were approximately the same between the residual and normal spleen. The results suggest that the tissue remodeling is dominated by vascular proliferation and lymphoid hyperplasia, and apoptosis is relatively inhibited in splenomegaly. With improvement in the tissue high pressure and hypoxic environment, the hyperplasia phenomenon reversed, the activity of cells renewed metabolism, the apoptotic state of inhibition was restarted, and the apoptosis levels of normal and residual spleen returned. This study is in agreement with the literature (Bax inhibitor-1 had high expression, the splenic apoptotic ratio decreased, and MØ uptake and cleaning apoptotic lymphocyte decrudescence was noted in apolipoprotein E mice with splenomegaly) [25-27]. Lan et al. [28] reported that in rats with pulmonary hypertension, the expression of hypoxia inducible factor-1α (HIF-1α), Bcl-2, and Bcl-xl increased, which suggests that there is a correlation between hypoxia, high pressure, and apoptosis. Yan et al. [29] pointed out that the factor inhibiting HIF1 (factor inhibiting HIF-1, FIH1) is an asparaginyl hydroxylase, which regulates the transcriptional activity of FIH1. Bax is an FIH1-specific contactor, and the FIH1 interaction with Bax for apoptosis is inhibited. The results of our study showed that the apoptosis is active in the residual spleen, and it is beneficial with respect to the anti-proliferative effect [30]. The results of our study and the study of Li et al. [31] is in contrast (after combined pancreas-spleen transplantation, the apoptotic ratio was significantly increased in recipients with splenomegaly). Using anti-Bax and anti-Bcl-2 staining, decreased positive expression of Bcl-2 proteins and increased positive expression of Bax proteins were detected in the residual and normal spleen, while increased positive expression of Bcl-2 proteins and decreased positive expression of Bax proteins were also observed in splenomegaly. The results showed that Bax/Bcl-2 ratios regulated apoptosis. When the Bax/Bcl-2 ratios increased, apoptosis was promoted; when the Bax/Bcl-2 ratios decreased, apoptosis was inhibited [32]. The results of our study confirmed splenocyte cycle updating, in which apoptosis in splenomegaly due to portal hypertension and residual spleen was achieved via the mitochondrial pathway [33-35]. This finding
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differs from the change in apoptosis level in splenomegaly caused by infection (increased apoptosis). Further, we showed that this molecular mechanism of apoptosis may be related to an exogenous pathway (FasL-mediated pathway) [36-38]. Electron microscopic observations confirmed that ECs, LYMs, and MØs in the residual spleen, control, and splenomegaly groups exhibited apoptotic morphologic features, such as fuzzy mitochondrial cristae, medullary changes, and margination of the nuclear chromatin. The results of our study are in agreement with the findings of others. The ultrastructural changes of apoptosis in splenic injury caused by chemical factors (cadmium and perfluorooctanoate poisoning) are different [39, 40].

Apoptosis is a multifactorial phenomenon, and there exist multiple death pathways inducible by different stimuli that can be positively or negatively regulated by the members of the expanding Bcl-2 family. Although Bcl-2 and Bcl-xl are negative modulators, rescuing cells from undergoing programmed cell death, Bax and Bcl-xs are inducers of apoptosis. Bax and Bcl-xs are able to form homodimers (Bax/Bax) and heterodimers (Bax/Bcl-2) in vivo that are associated with cell death and cell survival, respectively. The tissue inflammatory response involves complex interactions among inflammatory cells (neutrophils, lymphocytes, monocytes, and MØs), ECs, and extracellular matrix. Cytokines are produced by MØs, T-cells, and monocytes, as well as platelets and ECs. Circulating cytokines interact with specific receptors on various cell types and activate Janus kinase-signal transducers and activators of transcription (JAK-STAT), nuclear factor-κB (NF-κB), mitogen-activated protein kinase (MAPK), and Drosophila mothers against decapentaplegic protein (Smad) signaling pathways, thus leading to an inflammatory response involving cell adhesion, permeability, and apoptosis. Cytokines also interact with integrins and matrix metalloproteinases and modify extracellular matrix composition [41]. Persistent increases in cytokines are associated with splenic dysfunction and splenic disease, such as splenomegaly and hypersplenism. Therefore, our results confirmed dysregulation of apoptosis via the intrinsic pathway in splenomegaly. This observational study implicates apoptotic down-regulation in the spleen as a cause of splenomegaly, but does not exclude the possibility that other mechanisms are also responsible.

In conclusion, apoptosis is a multifactorial phenomenon. The different stimulation (inflammatory cells and inflammatory cytokines) can produce multiple signal pathways. Inflammatory cytokines can activate the JAK-STAT, NF-κB, MAPK, and Smad signal pathways, and can also interact with integrins and the matrix metalloproteinases signal pathways. Our results showed that splenomegaly and residual spleen after subtotal splenectomy due to portal hypertension can lead to different dysregulation of apoptosis via the mitochondrial pathway, which regulates the apoptosis priming protein (Bax or Bcl-2) and suggests that apoptotic down-regulation in splenomegaly and up-regulation in residual spleen, but does not exclude the possibility that other mechanisms are involved. Our study detected that the apoptosis level in the residual spleen approached the normal spleen. Meanwhile, we combined with our previous study indices [42-45], and may conclude that spleen-preserving surgery of splenomegaly in clinic have certain feasibility.

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Disclosure of conflict of interest

None.

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References

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