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Original Article

Changes in nerve fibers and microvessel density in residual spleen after subtotal splenectomy due to portal hypertension

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Abstract: Background: The precise pathogenic mechanism leading to splenic enlargement in patients with portal hypertension is poorly characterized. The present study was designed to determine the expression of nerve fibers and different angiogenic factors in patients with splenomegaly and residual spleens after subtotal splenectomy. Methods: Thirteen cases of splenomegaly due to portal hypertension were examined post-splenomegaly and designated as the splenomegaly group. During the ensuing 8 years we obtained other specimens by puncture biopsy, which were designated as the residual spleen group. We designated 13 patients with traumatic spleen injuries as the control group. Immunohistochemistry and immunofluorescence were used to determine the expression of neuropeptide Y (NPY), neurofilament-200 (NF-200) vascular endothelial growth factor (VEGF), vascular endothelial growth factor receptor-2 (VEGFR-2), and CD34 in residual spleens under light microscopy. Results: A significantly higher density of NPY- and NF-positive nerve fibers were observed in the splenomegaly group compared with the residual spleen and control groups (all $P<0.05$). Moreover, there were no significant differences between the residual spleen and control groups ($P>0.05$). A significantly higher microvessel density and positive expression ratio of VEGF and VEGFR-2 were observed in the splenomegaly group compared with the residual spleen and control groups (all $P<0.05$). There were also significant differences between the residual spleen and control groups ($P<0.05$). Conclusion: The residual spleen can eliminate the high-pressure state after a subtotal splenectomy, and remodeling of splenic nerves and microvessels with other splenic tissues and cells, which contributes to the recovery of splenic function.

Keywords: Residual spleen, portal hypertension, splenomegaly, nerve fibers, microvessel density

Introduction

It is well-known that the spleen has a central role in regulating the nervous and immune systems. Thus, a number of scholars have an interest in the distribution and regulation of splenic nerves [1-4]. The sympathetic arm of the autonomic nervous system may play a major role in regulating immune function via direct sympathetic innervation of all immune organs. The spleen is a model organ to study neural-immune interactions because of its well-described innervation and the ability to eliminate nerve fibers to the spleen by chemical or surgical sympathectomy. Analysis of the effects of neural transmitters on splenic immune function indicates a functional role for norepinephrine and neuropeptide Y (NPY) [5]. NPY, which has an important role as a neurotransmitter, is released by noradrenergic neurons along with norepinephrine. NPY, one of the most abundant neural peptides, exerts a great variety of physiologic actions, including the regulation of cardiovascular functions and food and water intake. In addition, NPY has been found in the nerve endings of immunocompetent organs, and NPY receptors are present in peritoneal murine macrophages, as well as in splenic cells [6]. Neurofilaments (NFs) are intermediate filaments of the neuronal cytoskeleton that provide the mechanical stability of cells and have a fundamental role in axonal transport [7]. While NFs are present in dendrites and perikarya, NFs are abundantly present in axons, maintaining the structural integrity and caliber of axons by

influencing the conduction velocity of nerve impulses [8]. After repeating electric stimulation to splenic nerves, secreting of splenic cytokines was downregulated, which leaded to immune dysfunction [9, 10]. The recovery of splenic nerves after splenic auto-transplantation ensured the integrity of splenic structure and function [11, 12].

More recently, the role of tissue hyperplasia, including not only blood congestion in the splenic red pulp, but also a combination of angiogenesis and fibrogenesis, and enlargement and hyperactivation of the lymphoid compartment of the spleen in addition to congestion, has also been suggested [13]. Recent studies have highlighted that angiogenesis, the growth of new blood vessels from a pre-existing vascular bed, is a pathologic hallmark of portal hypertension. Thus, increased splanchnic neovascularization regulated through the coordinated action of vascular endothelial growth factor (VEGF) and platelet-derived growth factor has been demonstrated in experimental models of portal hypertension and has been shown to be a crucial mechanism by which portal hypertension, hyperdynamic splanchnic circulation, and portosystemic collateralization are initiated and stabilized [14-20]. Other results demonstrate significant up-regulation of the powerful angiogenic growth factor, VEGF, and the neovascularization markers, vascular endothelial growth factor receptor-2 (VEGFR-2), CD34, a-smooth muscle actin (α-SMA), and platelet-derived growth factor receptor-beta (PDGFR-β) in the spleen from portal hypertensive rats [21]. The splenic microvessel density (MVD) was increased in the early stage after splenic auto-transplantation, and accompanied by the positive expression of VEGF and VEGFR-2 [22]. In the fetal spleen, VEGF has been shown to have a positive expression in smooth muscle cells and pericytes of splenic central arteries and veins, while there was no positive expression in splenic sinuses [23].

The distribution of nerves and microvessels in residual spleen after subtotal splenectomy has not been reported. The current study detected and analyzed the positive expression of NPY, NF200, VEGF, VEGFR-2, and CD34 in the spleen by immunohistochemistry and immunofluorescence, and assessed the distribution of nerves and microvessels to provide a theoretical basis for 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 to be involved in the study.

Specimen collection and processing

Our studies relating to subtotal splenectomy began in 1984. As of 2015, we have conducted 852 subtotal splenectomies (preserving the lower pole, normal spleen 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. 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 Doppler ultrasound was used before surgery and 8 years after surgery to measure the size of the spleen. The preoperative 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 sple-

nectomies, but who did not have splenomegaly, comprised the control group.

Experimental methods

Paraffin sections were made. Thirty-nine tissue samples were collected. Specimens were fixed in 10% formalin, dehydrated, embedded, and sliced. Each specimen was cut into 15 slices, with 5 slices per group. These sections were used for immunohistochemistry and immunofluorescence staining.

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 primary antibody (rabbit anti-human NF200 and NPY monoclonal antibodies were used to label the nerve fibers; rabbit anti-human VEGF and VEGFR-2 monoclonal antibodies were used to label the corresponding antigens; and rat anti-human CD34 monoclonal antibody was used to label the endothelial cells), followed by incubation at room temperature for 30 min with biotinylated secondary antibody with diaminobenzidine (DAB) 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 (PBS). NPY was purchased from Biosynthesis Biotechnology Co., Ltd. (Beijing, China) and NF200, CD34, VEGF, and VEGFR-2 were purchased from Zhongshan Golden Bridge Co., Ltd. (Beijing, China).

Immunofluorescence staining

The paraffin sections were dewaxed, twice-dehydrated in xylene for 10 min, then hydrated in 100%, 95%, and 80% ethanol for 2 min. A 0.01 M citrate buffer solution (pH 6.0) was added to the sections dropwise, then microwaved at 500 W for 3 min and cooled rapidly. The sections were thrice-washed in PBS for 5 min, and sealed with sheep serum at 37°C for 30 min. NF200 and NPY reagent was added dropwise at 37°C for 1 h, then thrice-washed in PBS for 5 min and protected from light. Finally, the sections were mounted with glycerol PBS. The nucleus of positive cells exhibited a yellow-green fluorescence under a fluorescence microscope. Sheep serum was purchased from Biosynthesis Biotechnology Co., Ltd. NF200 and NPY reagents were provided by Biosynthesis Biotechnology Co., Ltd. The microwave buffer solution was self-made, but the reagent was provided by Beijing Chemical Factory (Beijing, China).

Criterion of immunostaining 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. Under light microscopy (×400), Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA) was used to calculate the density of NPY- and NF200-positive nerve fibers (fibers/mm^2), the expression ratio of VEGF and VEGFR-2 positive cells (%) and the MVD, respectively. Five random fields per section from the same tissue samples were counted and the mean was calculated [24, 25]. The positive expression ratio of VEGF and VEGFR-2 positive cells = (number of positive cells/number of total cells [per high power field]).

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 Student's t-test with two independent samples and two paired samples. A $P<0.05$ was considered significant.

Results

Distribution of NF200- and NPY-positive nerve fibers in spleens

The results obtained with the immunohistochemical assay were the same as the results obtained with the immunofluorescence assay. In the white pulp, positive nerve fibers were densely distributed surrounding the central artery and its branches and periarterial lymphatic sheath. In the red pulp, scattered positive nerve fibers were distributed around the wall of the splenic sinusoids and small blood vessels. Scattered positive nerve fibers were present in the marginal sinus and marginal zone, as well as the splenic capsule and spleen trabecula. The distributions of positive nerve fibers were similar among the three groups, but the splenomegaly group showed a relatively high density (**Figure 1A-L**).

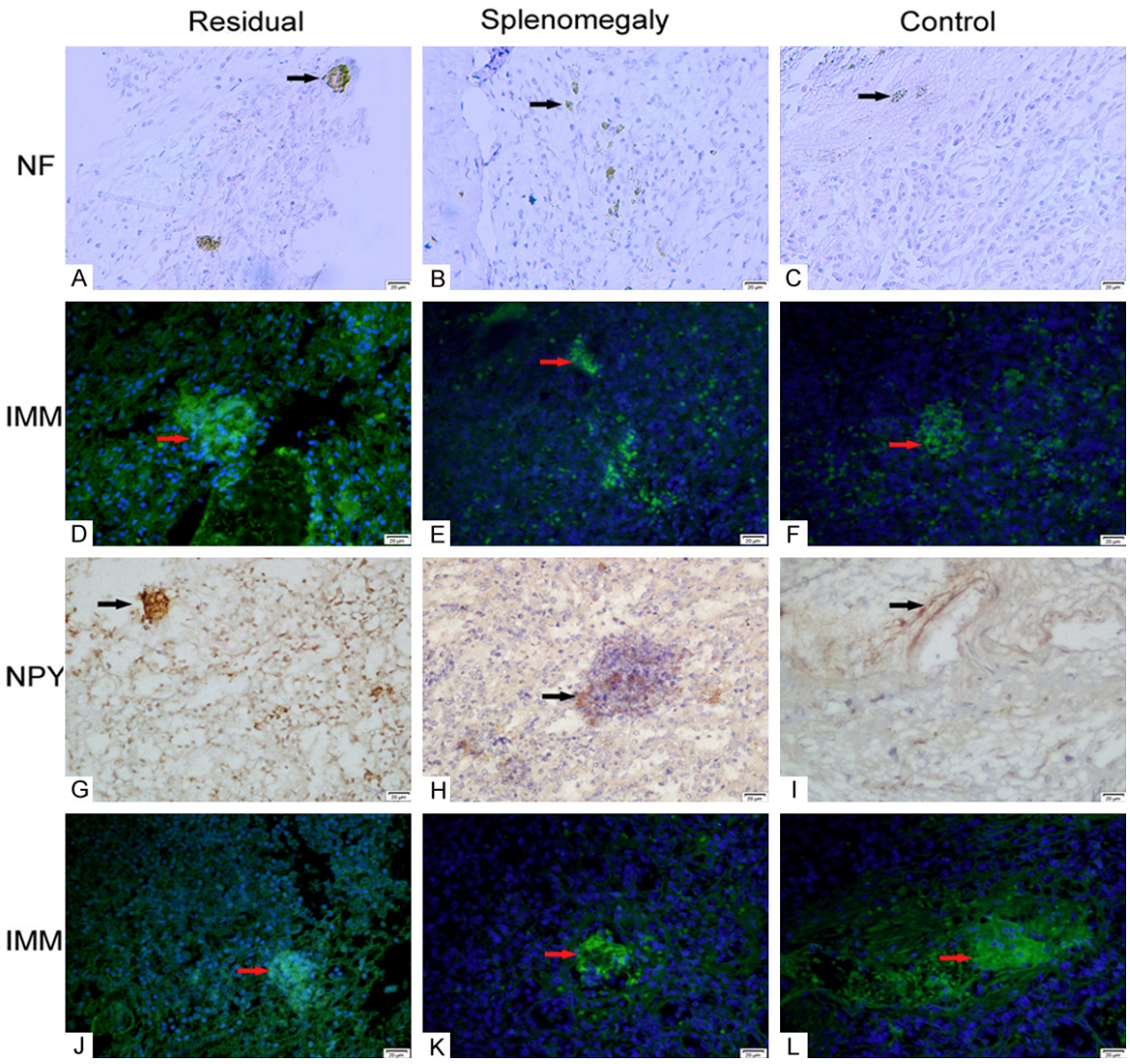

Figure 1. The positive expression of nerve fibers in the residual spleen (A, D, G, J), the splenomegaly spleen (B, E, H, K), and normal spleen (C, F, I, L). (the arrow indicates positive expression, immunohistochemistry and immunofluorescence, ×400 magnification, bar = 20 μm).

Density of NF200- and NPY-positive nerve fibers in spleens

The densities of positive nerve fibers in the splenomegaly group were significantly higher than the residual spleen and control groups (all $P<0.05$), while there was no significant difference between the residual spleen and control groups ($P>0.05$, **Figure 2A**).

Distribution of positive expression of VEGF, VEGFR-2, and CD34 in spleens

In the three groups, only scattered positive cells of VEGF and VEGFR-2 were observed in the marginal zone and red pulp. The expression of VEGF was mainly localized in macrophages, and the expression of VEGFR-2 was mainly localized in endothelial cells (**Figure 3A-F**).

In the residual spleen and splenomegaly groups, the positive cells of CD34 were densely distributed in the red pulp. In the control group, scattered positive cells of CD34 were presented in the red pulp. In the three groups, scattered positive cells of CD34 were observed in the marginal zone, white pulp, and spleen trabecula (**Figure 3G-I**).

Quantitative analysis of positive expression of VEGF, VEGFR-2, and CD34 in spleens

A significantly higher MVD and positive expression ratio of VEGF and VEGFR-2 were observed

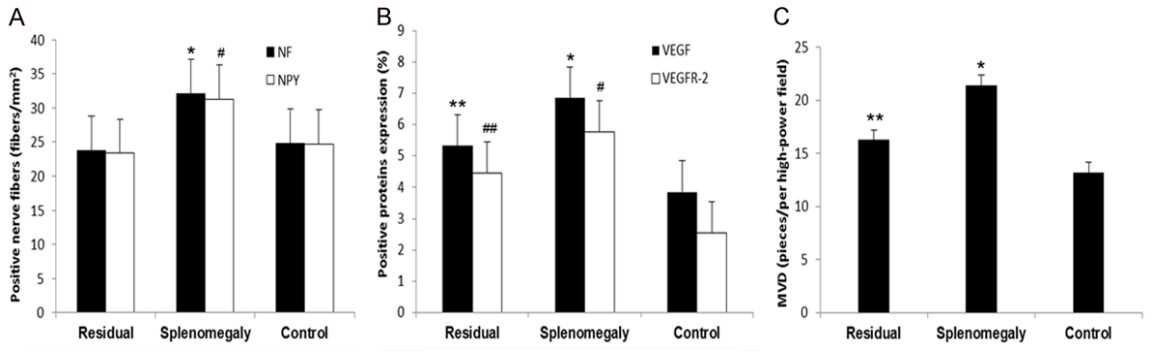

Figure 2. Density of NF and NPY in the residual spleen, the splenomegaly spleen, and normal spleen (A. fibers/mm^2, *P<0.05, #P<0.05); positive expression ratio of VEGF and VEGFR-2 proteins in the residual spleen, the splenomegaly spleen, and normal spleen (B. %, *P<0.05, #P<0.05, **P<0.05, ##P<0.05); MVD of CD34 in the residual spleen, the splenomegaly spleen, and normal spleen (C. pieces/per high-power field, *P<0.05, **P<0.05).

in the splenomegaly group compared with the residual spleen and control groups (all P<0.05). The residual spleen group was also significantly higher than the control group (P<0.05, **Figure 2B**, **2C**).

Discussion

Ninety-eight percent of nerve fibers in rat spleens are sympathetic nerves, which arise from the mesenteric superior celiac ganglion. Post-ganglionic noradrenergic plexiform fibers enter the spleen along the splenic artery via the splenic hilum, and extend to the splenic trabecula [26, 27]. The plexiform nerve fibers of splenic vessels and trabecula could enter around the central artery and its branches of the white pulp. Noradrenergic neuronal somas arise from the periarterial lymphatic sheath (APLS) nerve plexus, and the density is related to the central artery of the white pulp and APLS. Noradrenergic fibers are sparsely distributed in the marginal zone, venous sinus, and parafollicular zone. Those point- or linear-shaped nerve fibers are densely distributed around specific cells of the splenic white pulp and marginal zone [28]. Splenic noradrenergic fibers are closely related to the lymphocytes and other immune cells in the white pulp. Scattered nerve fibers in red pulp are related to the splenic trabecula and nerve plexus in peripheral tissues [29, 30]. The splenic NPY-positive nerve fibers could reflect the distribution of noradrenergic fibers, which possess the characteristics of common regionalization between NPY and noradrenergic fibers. The terminal NPY-positive nerve fibers contact T lymphocytes and macrophages of APLS in white pulp, the OX8-positive T lymphocytes of the nerve plexus around the splenic trabecula in red pulp, and the immunoglobulin M-positive lymphocytes and ED1/ED3-positive cells in the marginal zone, which have been confirmed by electron microscopy [31].

Therefore, clarifying the structure of splenic nerve anatomy contributes to defining the relationships between nerves and immunocytes, as well as the effect on splenocytes and splenic function by neurotransmitters.

CD34 is a type of transmembrane glycoprotein with a molecular weight of 110 kDa, which was expressed in endothelial cells and cells of the splenic marginal zone. VEGF is a multi-functional cytokine and a specific vascular growth factor which was isolated from bovine pituitary follicle cell culture medium by Ferrara et al. in 1989 [32]. VEGF is an endothelial cell mitogen with multi-functions, including enhancement of the viability of endothelial cells, promoting mitosis, inducing angiogenesis, promoting cell migration, and inhibiting apoptosis [33]. In the early 1990s, Terman et al. [34] isolated VEGFR-2 from an endothelial cell bank from human umbilical veins. It has been demonstrated that VEGFR-2 occurs in vascular endothelial cells, with the following functions: inducing mitosis; proliferation; transference; regulating the permeability of endothelial cells; and participating in the process of angiogenesis [35]. VEGF exerts a biology effect by binding to its corresponding receptor on the cell membrane [36]. In a pathologic state, abnormal expression of VEGF and hypoxia can induce the up-regulation of VEGF expression, and combine with its specific receptor, VEGFR, followed by a series of

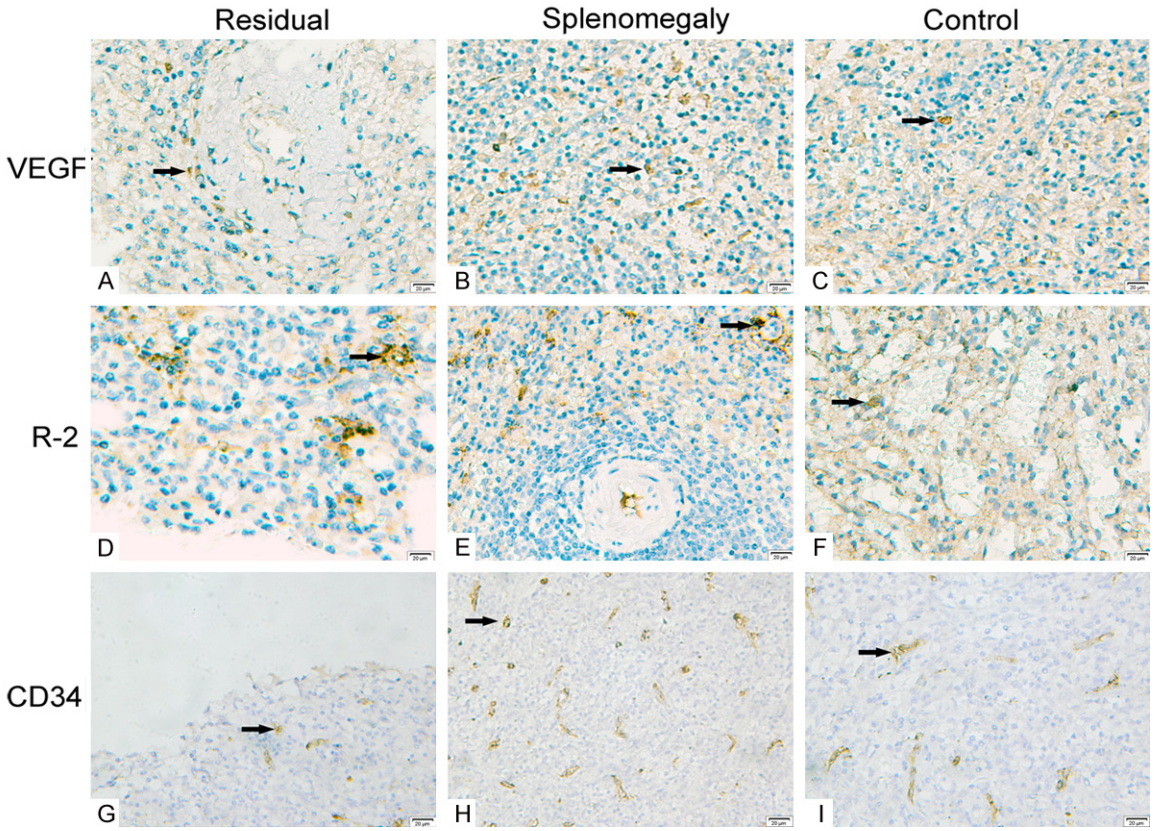

Figure 3. The positive expression of VEGF, VEGFR-2 and CD34 in the residual spleen (A, D, G), the splenomegaly spleen (B, E, H), and normal spleen (C, F, I). (the arrow indicates positive expression, immunohistochemistry, ×400 magnification, bar = 20 μm).

signal transductions, which could strongly induce mitosis and proliferation of endothelial cells, stimulating hyperplasia of vascular endothelial cells and an increase in vascular permeability, promoting angiogenesis, and improving the blood supply of tissues [37]. In splenomegaly tissues, the expression of VEGF and platelet-derived growth factor were high [38, 39]. Therefore, exploring the expression of CD34, VEGF, and VEGFR protein levels in the spleen could help to understand the relationship between splenomegaly MVD and angiogenesis and the underlying mechanism.

NPY and NF were sensitive nerve markers presenting in sympathetic nerve axons. Meanwhile, NPY also had a role as a neurotransmitter to regulate cellular immune functions by adhesion, chemotaxis, phagocytosis, lymphoid tissue hyperplasia, producing antibodies and cytokines [40, 41]. Straub et al. [42] found that NPY-positive nerve fibers are observed in the wall of the central artery of the white pulp and APLS in NMRI rats, which indicated that neurons had a fine-tuning effect on macrophages because nerve-immune cells respond to noradrenaline and NPY is a modulator of neuroplasticity, neurotransmission, and memory. Animal studies have shown that rat splenic NPY-positive nerve fibers are densely distributed in the vessels of the splenic hilum and around the central artery and its branches in the white pulp, while in the veins and venules of the red pulp and splenic trabecula and capsule they have a scattered distribution [31]. Other scholars have reported that the distribution of porcine splenic chromogranins and NPY-positive nerve fibers were co-localized. Positive nerve fibers formed a dense plexiform around central small arteries and its branches in the white pulp. Only a few positive nerve fibers were found in the walls of arterioles and venules of red pulp and in the splenic capsule. In contrast, positive chromogranins and NPY were not observed in splenocytes [43]. Anagnostou et al. [44] discovered massive NF-positive fibers

around the central artery and its branches in the white pulp and scattered fibers of red pulp in the fetal spleen. Feng et al. [45], using western blotting and immunohistochemistry, indicated that NF-positive fibers were distributed in the splenic capsule and trabecula, as well as arterial branches, and the arterial periarterial lymphatic sheath was also observed as a slender, dense nerve plexus.

With respect to the expression of VEGF and VEGFR-2 in splenic tissues, there were differences in time and space in the research results. Zhang et al. [22] found that the MVD reached the highest value in 180 days, and the densities of VEGF- and kinase insert domain receptor (KDR)-positive cells reached the highest value 60 days after splenic auto-transplantation. The rat splenic MVD was higher in the sham operation group than the splenic slices transplantation group, but the densities of VEGF- and KDR-positive cells were lower. Radwan et al. [46] reported that the genetic expression of angiogenic, pro-inflammatory, pro-fibrogenic cytokines was increased in patients with splenomegaly. Research has shown that in the normal human spleen, macrophages and small vessels KDR protein (analogue of human VEGFR-2) had a positive expression, while lymphocytes had a negative expression [47]. Another study showed that the human VEGF-D mRNA had high expression in skeletal muscle, skin, pancreas, heart, and spleens of transgenic mice. The MVD of skeletal and cardiac muscles was increased, while there was no change in the density of lymph capillaries [48]. Joory et al. [49] found that VEGF-C mRNA and protein had a positive expression in many neoplastic tissues, but there was no positive expression in the thymus and spleen. Animal experiments confirmed that the level of VEGF in portal hypertensive rats was higher than the sham operation group, which was quantitatively detected by western blotting [38]. The results indicated a possible underlying mechanism, namely the congestive spleen can be in a state of low oxygen, and tissue hypoxia might be an important factor that stimulates the expression of VEGF [50]. Melgar-Lesmes et al. [51] indicated that the VEGF-A of portal hypertensive rats had a higher expression in the mesenterium and liver, while there was no obvious change in the kidney and spleen compared with the control group. The positive expression of mesenteric CD31 was in accordance with VEGF-A [16].

Our study showed that the distribution of NPY- and NF-positive nerve fibers in the three groups were the same, while the densities of NPY- and NF-positive nerve fibers were highest in the splenomegaly group, and there was no significant difference between the splenomegaly and control groups. Our results agreed with previously published results [43, 45]. Nerve tissues were the same with other tissues and cells in the spleen, which were a part of splenic structure and function. In the residual spleen, dense NPY- and NF-positive nerve fibers were observed in the central artery of the white pulp and APLS, but only scattered positive fibers were found in the marginal zone, red pulp, splenic capsule, and trabecula, which was consistent with the distribution regularities of splenic sympathetic nerves (noradrenergic nerves). The number of NPY and NF determined the diameter of the axon, and the expression intensity of the area could reflect the degree of neural network construction perfected. With a decrease in NPY- and NF-positive expression, the decline in NF activity, followed by maintaining normal structure and function of nerve cells, indicated that the neural network structure was destroyed [52]. As almost all of the splenic nerves contained noradrenergic fibers, we chose NPY and NF as neural-sensitive markers. Under normal circumstances, NPY and NF are only observed in axons, there was no expression in neuronal soma. When nerves are injured, positive expression is present in the neuronal soma [53]. This might be the reason that only NPY- and NF-positive nerve fibers were found in our study. Romano et al. [31] reported that in normal rat spleens, a few NPY-positive cells were in lymphocytes of peripheral splenic nodules and macrophages near the splenic trabecula. However, we could not find NPY-positive cells in any part of the human spleen, which might be concerned with species variation or a different amount of NPY in neurons. In our previous experiments, we found that compared with pre-operative splenomegaly, the fibrous tissue of residual spleen 8 years post-operatively had no apparent change, but a significantly higher number of T lymphocytes, B lymphocytes, and macrophage per unit area. The number of splenic corpuscles and APLS approached the number in a normal splenic sinus, congestion disappeared, and decreased the density and the average cross-sectional area of the penicillar artery were observed in

residual spleen compared with splenomegaly [54, 55]. These results implied that there was no change in the degree of fibrosis in the residual spleen, but the number of splenic small vessels and immune cells per unit area were close to normal. Our initial clinical follow-up observed that compared with pre-operative values, the white blood cell and platelet counts were higher post-operatively, the serum levels of macrophage colony-stimulating factor and granulocyte-macrophage colony-stimulating factor were significantly decreased, the proportion of serum CD3+ T cells, CD4+ T cells, and CD8+ T cells was increased, and there was no significant change in the serum levels of IgA, IgM, IgG, and Tuftsin. Isotope scanning with 99mTc showed that the residual splenic phagocytic function had recovered post-operatively [56]. Based on our own research in the changes of nerve fibers and MVD, we conclude that the function of the residual spleen is the same as normal spleen.

Our study also showed that immunohistochemical staining of VEGF, VEGFR-2, and CD34 showed a significantly higher MVD and positive expression ratio of VEGF, VEGFR-2, and CD34 was observed in the splenomegaly group compared with the residual spleen and control groups, and the residual spleen group was also higher than the control group. Our results suggest that microvascular proliferation existed in splenomegaly, but was not abundant in other organs (placental tissues) and tumor tissues [57, 58]. In patients with splenomegaly, the tissues showed a state of high pressure and chronic passive congestion, which could promote the expression of VEGF and VEGFR-2 and become the initial factors of revascularization [59]. Millauer et al. [60] reported that VEGF is a potent mitogen of cells, with a high affinity for VEGFR-2, and showed consistency of space and time between VEGFR-2 and VEGF. There was a high expression of VEGF, VEGFR-2, and CD34 in malignant tumors, but it has a low expression in most normal tissues and benign tumors [61]. Tumor cells have the function of secreting VEGF, promoting microvascular abnormal growths, which might be one of the important reasons for a positive expression of VEGF and VEGFR-2 in tumor tissues [62]. Research showed that KDR existed in a variety of vascular endothelial cells in the embryonic period, but was reduced in the mature period [63, 64]. Thus, results of the VEGF- and VEGFR-2-positive expression in the embryonic development period suggested that the increase in positive expression of VEGF and VEGFR-2 in splenomegaly and residual spleen might be related to the static or resting tissues or cells in normal spleen [65].

Conclusion

The residual spleen can eliminate the high-pressure state after subtotal splenectomy, and the remodeling of splenic nerves and microvessels is the same with other splenic tissues and cells which contributes to the recovery of splenic function (the correlation between splenic nerves and immune cells increased [66], and the microvessel density decreased). Our study confirmed that there is a consistency of space and time between VEGF and VEGFR-2, and the change in microvessels was closely related to the demand of tissue metabolism. The pluralism of splenic nerves (noradrenergic nerves, cholinergic nerves, peptidergic nerves, and growth-associated protein nerves) were demonstrated the complexity of neurotransmitter and receptor [67, 68]. With respect to relevance between splenic nerve endings and immune cells under an electron microscope, as well as the acting mode of hormone and neuropeptide to immune cells, these problems need further study.

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Disclosure of conflict of interest

None.

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