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Sympathetic nervous system mediates surgical trauma stress-induced splenocyte apoptosis in rats

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Abstract

Surgical trauma stress has been reported to induce immunosuppression. The mechanisms involved are still unclear. The present study was designed to assess the role of the sympathetic nervous system in regulating splenocyte apoptosis induced by surgical trauma stress. Our results showed that the rats that underwent surgical trauma stress exhibited a significant reduction in splenic cellularity, the loss of splenocytes was likely mediated by apoptosis, for a substantial increase in apoptosis was observed by using DNA gel electrophoresis and TUNEL assay. At the same time, an increase in Fas(CD95/Apo-1) protein expression in splenocytes was also observed. These effects were significantly abolished by either chemical sympathectomy or beta-adrenergic receptor antagonist propranolol. The data clearly revealed that the sympathetic nervous system especially beta-adrenergic receptors was involved in surgical trauma-induced immune alterations via a mechanism of apoptotic cell death.

Keywords: Sympathetic nervous system; Splenocyte apoptosis; Surgical trauma; Stress; Beta-adrenergic receptor; Propranolol

1. Introduction

Several studies both in the human and in the animal have shown that surgery is associated with immunosuppression (Allendorf et al., 1997; Berti Riboli et al., 1984; Gupta, 1987; Nelson et al., 2000). The immunodepression observed following surgical procedures is widespread and makes the host vulnerable to infection and disease. Research has shown that surgery depresses several aspects of immune functions, such as decreased splenocyte proliferation to concanavalin A, depressed antigen presentation, impaired microbial activity, decreased natural killer cell activity, and reduced production of a number of cytokines. These results clearly show that surgery causes profound changes on the immune system. Previous studies in our lab also revealed that a severe stress induced by surgical trauma resulted in immunosuppression and disturbance of neuroendocrine system (Cheng et al., 1997; Du et al., 1998; Zhang et al., 1996). The surgical trauma could significantly suppress splenic lymphocyte proliferation; concanavalin A induced interleukin-2 production and natural killer cell activity.

Although it is clear that surgery produces immune alterations in both human and animal, there continues to be plenty of investigation of the mechanism involved in these effects. Apoptosis is an evolutionary conserved "cell suicide" program present in all nucleated metazoan cells. In the immune system, apoptosis occurs physiologically during lymphocyte repertoire selection and immune responses to various diseases. Lymphocyte apoptosis also characterizes severe pathophysiological states including thermal injury, sepsis and surgery in humans (Kono et al., 2001; Oka et al., 1996). But little is known about the effect of surgical stress on lymphoid organ. Interestingly,

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our previous studies found that rats underwent surgical trauma stress may result in a substantial increase in splenocyte apoptosis (Wang et al., 2005), but the mechanisms involved in the process is undiscovered.

Accumulated studies have shown that the neuroendocrine system may play an important role in perioperative immune depression. Recently in the studies of the physiological regulation of stress, besides the classical hypothalamic–pituitary–adrenal axis, more attention is paid to the locus coeruleus-noradrenaline mediated sympathetic nervous system (Elenkov et al., 2000). Lots of evidence revealed that sympathetic nervous system plays an important role in stress-induced immunomodulation (Madden, 2003; Nagatomi et al., 2000; Zhao et al., 1995). The relationship between the surgery induced inhibition of T and B cell proliferation and adrenergic stimulation has recently been demonstrated by using nalodol, a β-adrenergic receptor agonist (Nelson and Lysle, 1998). In spite of the evidence of the immunosuppressive effects of adrenergic substance, sympathetic nervous system is associated with splenocyte apoptosis in surgical stress was seldom mentioned. Since the spleen is innervated by sympathetic nerve and the beta-receptor exists on the splenic lymphocyte (Sanders et al., 2001), the chemical sympathectomy and beta-receptor antagonist propranolol were chosen to address their role in surgical trauma stress.

It has been documented that Fas/FasL (CD95/CD95L) system is an important factor in regulation of apoptotic cell death in the splenocyte development under normal condition and in response to various insults, Fas/FasL system also plays an integral role in maintaining cellular homeostasis of the immune system (Krueger et al., 2003). Therefore, the aims of this study were to determine: 1) whether the surgical trauma stress-induced splenocyte apoptosis was associated with sympathetic nervous system; (2) whether increased levels of apoptosis were correlated to the regulatory factor, Fas antigen.

2. Materials and methods

2.1. Traumatic animal model

Male Sprague–Dawley rats weighing 200–220 g were obtained from the Experimental Animal Center of Shanghai Medical College, Fudan University. The animals were acclimatized to the experimental conditions for 1 week before the experiment in order to reduce any nonspecific influence. The surgery was performed as previously described (Zhao et al., 2002). Briefly, rats were anesthetized with 35 mg/kg Nembutal administered intraperitoneally (i.p.). Animals were then incised longitudinally to a length of 6 cm along the dorsal median line and 5 cm along the abdominal median line. After surgery, the wounds were sutured and the animals were kept warm under standard housing conditions and allowed free access to standard rat pellet diet and tap water. No post-operative infection occurred. Animals were divided into six groups: (1) control group: no manipulation was made; (2) trauma group: standard abdominal surgery was performed; (3) trauma + sympathectomy group: chemical sympathectomy was performed by 6-hydroxydopamine (6-OHDA, Sigma) injections according to the method described by McCafferty (McCafferty et al., 1997). The rats were injected subcutaneously with four doses of 6-OHDA dissolved in 0.9% NaCl and 0.1% ascorbic acid within 7 days (50 mg/kg on day 1 and day 2, and 100 mg/kg on day 6 and day 7). Surgical trauma stress was performed on day 8 under anesthesia; (4) trauma + propranolol group: propranolol (Sigma, i.p. 10 mg/kg) was injected 30 min before surgical trauma. In stressed group, same volume of the vehicle was injected instead of propranolol; (5) no surgery + sympathectomy group; chemical sympathectomy was carried out on normal rats; (6) no surgery + propranolol group. Each group consisted of six rats. All protocol presented in the study was approved by the Committee on Research Animal Care of Fudan University, and followed by the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals.

2.2. Single cell preparation

The rats were decapitated 24 h after surgical trauma. A single-cell suspension of splenocytes was prepared by using the blunt end of a syringe and passing the suspension through nylon gauze. Red blood cells were lysed with lysing buffer. Splenocyte numbers were determined by counting cells by using hemocytometer. Isolated splenocytes were adjusted to 5×10^6 cells/ml in RPMI 1640 supplemented with 10% FCS, 10 mM Hepes, 1 mM glutamine and 50 mg/ml penicillin.

2.3. DNA isolation and fragmentation detection

The "ladder pattern" of DNA fragmentation was detected by agarose gel electrophoresis. Briefly, the splenocytes were cultured for 24 h at 37 °C in 5% CO_2 incubator at a concentration of 5×10^6 cells/ml in RPMI 1640. The cultured suspending cells were harvested by centrifuged at 180 g for 5 min, and washed by 0.01 M PBS twice. Then the splenocytes were resuspended in 0.5 ml digestion buffer (100 mM NaCl; 10 mM Tris–HCl, pH 8.0; 25 mM EDTA, pH 8.0; 0.5% SDS; 0.2 mg/ml proteinase K) and incubated at 50 °C overnight for cell digestion. The digested cell samples were extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) solution. The DNA was precipitated by 2 volumes of cold 100% ethanol and isolated by centrifugation. After dried in air, the DNA pellet was resuspended in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). The DNA samples were loaded in 1.0% agarose gel containing ethidium bromide (0.5 μg/ml). After electrophoresis at 80 V for an h, the DNA bands were visualized under UV transillumination.

2.4. TdT-mediated dUTP nick end labeling (TUNEL) reactions on fixed spleen sections

Nucleosomal DNA fragmentation in spleens was determined by the TUNEL assay. For TUNEL reactions on tissue sections, paraffin-embedded spleen was processed as described for the in situ apoptosis detection kit (TA4625, R&D company). In brief, 5 μm tissue sections were performed deparaffinization first,

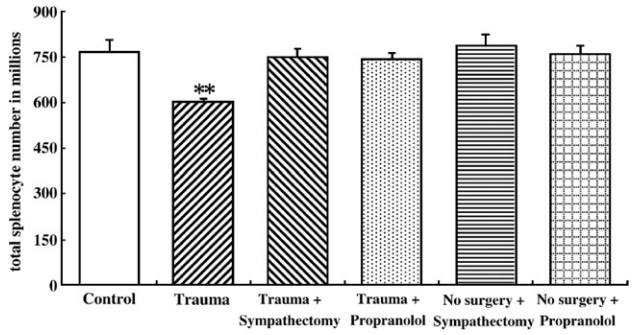

Fig. 1. Effects of surgical trauma stress on splenic cellularity. Control: non-stressed rats; trauma: surgical trauma stressed rats; trauma + sympathectomy: chemical sympathectomy was performed by 6-OHDA (Sigma) injections before surgical trauma. Trauma + propranolol: propranolol (i.p. 10 mg/kg) was injected 30 min before surgical trauma. No surgery + sympathectomy: 6-OHDA was injected to normal rats. No surgery + propranolol: propranolol was injected to normal rats. Data were represented as mean±S.E.M. ($n=6$ in each group). $^{**}P<0.01$ vs control group.

then incubated in permeabilization solution (Proteinase K digestion) for 30 min at room temperature. After that, the sections were incubated with quenching solution (0.3% H_2O_2 dissolved in methanol) for 5 min at room temperature following by washing in PBS. After adding 50 μl of TUNEL reaction mixture, slides were incubated in a humidified chamber for 120 min at 37 °C, then transferred to 50 ml stop buffer for 5 min, rinsed two times with PBS. Thereafter, slides were covered with 50 μl of the Streptavidin-Horseradish Peroxidase (HRP) Detection Solution for 20 min at room temperature, rinsed twice with PBS, and then were added with 50 ml of fresh DAB-substrate solution for 5 min at room temperature. Rinse the slides in 50 ml dH_2O, Methyl Green Counterstain were processed for spleen sections. Cover slips were mounted and tissue sections were examined under light microscopy with an Olympus microscope. For quantitative data, images of positive staining in the splenic sections were captured and analyzed using Leica Q500IW image analysis system. For each rat, three sections were used and ten randomly selected fields were examined for each slide. The investigator responsible for image analysis was blind to the experimental condition of each rat. The incidence of apoptotic cells was expressed as number of apoptotic cells/100 nuclei.

2.5. Western blotting for Fas protein

Splenic homogenate from each recipient rat was centrifuged at 15,000 *g* for 20 min twice, and the supernatant was used for Western blot analysis of Fas protein. Aliquots of supernatant containing 20 μg of protein were resolved in 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 12%). The separated proteins were then transferred (130 V for 1 h) onto Polyvinylidene Difluoride (PVDF) membrane. After incubated in Blocking reagent (1X TBS, 5% milk, 0.05% Tween-20) at 37 °C for 60 min to block unspecific binding, the membrane was incubated in Blocking reagent containing a rabbit polyclonal anti-Fas antibody Fas(M-20) (Santa Cruz Biotechnology, Santa Cruz, CA) in 1:200 dilutions at 37 °C for 1 h. After washing in TBS containing 0.05% Tween-20 (TTBS) buffer, the membrane was incubated for 30 min at 37 °C in TTBS buffer containing a 1:500 dilution of HRP-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA). To visualize the result, chemiluminescence reaction using the ECL system was adopted (Santa Cruz Biotechnology, Santa Cruz, CA).

2.6. Immunohistochemistry

For immunohistochemistry on tissue sections, paraffin-embedded spleen was processed as TUNEL assay. In brief, 5 μm tissue sections were performed deparaffinization first, then following three 15-min rinses in 0.01 M PBS, the sections were preincubated for 30 min at room temperature in a blocking solution of 3% normal goat serum in 0.01 M PBS with 0.3% Triton X-100 (NGST) and then incubated in primary antibodies (rabbit polyclonal anti-Fas antibody Fas(M-20) (1:200, Santa Cruz, Inc., USA), diluted in 1% NGST at 4 °C for 24 h. The incubated sections were washed three times in 1% NGST and incubated in biotinylated goat anti-rabbit immunoglobulin G (IgG) (1:200, Vector Laboratories, Burlingame, CA) for 1 h at room temperature. The sections were then washed three times in 1% NGST and incubated for 1 h in avidin–biotin–peroxidase complex (1:200, Vector Laboratories) at room temperature. Finally, the sections were washed three times in 0.01 M PBS, and immunoreactive products were visualized by catalysis of 3,3-diaminobenzidine (DAB) by horseradish peroxidase in the presence of 0.01% H_2O_2. The sections were then mounted, dehydrated and covered. To test the specificity of the primary antibody, controls were performed, including the substitution of normal rabbit sera for the primary antibody and omission of the primary antibody. None of these controls showed any sign of immunohistochemical reaction.

2.7. Data analysis

Data are presented as mean±S.E.M and analyzed by SPSS 10.0 software. One-way analysis of variance (ANOVA) was used for overall effects, with S–N–K test for post-hoc analysis for

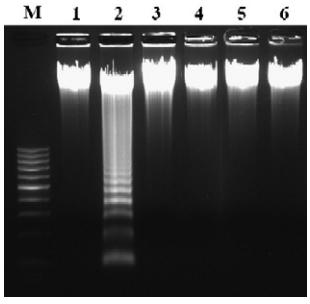

Fig. 2. DNA ladder in gel electrophoresis from control and traumatic rat spleen. DNA was isolated from the splenocyte of rats that underwent surgical trauma and given with either sympathectomy or β-adrenergic receptor antagonist propranolol before experiment. M: Marker for DNA size (200 bp each line); line 1: control; line 2: trauma; line 3: trauma + sympathectomy; line 4: trauma + propranolol; line 5: no surgery + sympathectomy; line 6: no surgery + propranolol.

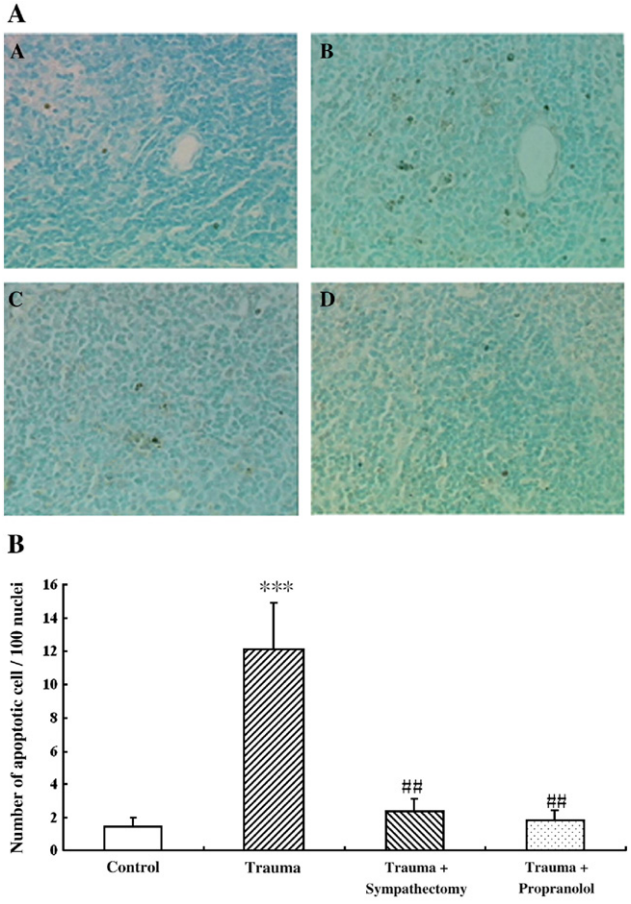

Fig. 3. Surgical trauma induces apoptotic cell death in rat spleen, as well as the quantitative evaluation of apoptotic cells. (A) TUNEL assay for apoptotic cells in rat spleen. TUNEL-positive apoptotic cells in spleen sections were shown in dark brown on a methyl green counterstained background. All photos were taken at 200×, A: control; B: trauma; C: trauma + sympathectomy; D: trauma + propranolol. (B) Quantitative data for stress-induced apoptotic cells observed by light microscopy with TUNEL staining. ***$P<0.001$ vs control group, ##$P<0.01$ vs trauma group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

differences between groups. $P<0.05$ was considered statistically significant.

3. Results

3.1. Effects of surgical trauma stress on splenic cellularity

We subjected rats to surgical trauma stress, 24 h later we found that this treatment dramatically affected splenic cellularity. The number of splenocytes dropped from 7.68±0.369 ($\times 10^8$) to 5.94±0.18 ($\times 10^8$) ($P<0.01$). These rats showed a 25–30% reduction in the number of splenocytes compared with unstressed controls (Fig. 1). The effects were antagonised in either sympathectomized rats or i.p. propranolol rats, the number of splenocytes were 7.49±0.264 ($\times 10^8$) and 7.42±0.228 ($\times 10^8$) respectively. Neither sympathectomy nor propranolol given to normal rats affects splenic cellularity, the number of splenocytes was 7.87±0.385($\times 10^8$) and 7.62±0.254 ($\times 10^8$) respectively (Fig. 1).

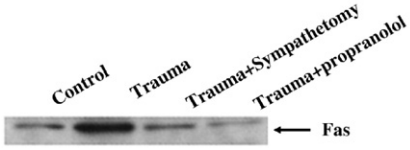

Fig. 4. Effect of surgical trauma stress on splenocyte Fas protein expression by using Western blot assay. The data are representative of six independent experiments.

3.2. Surgical trauma-induced apoptosis in rat spleen

DNA gel electrophoresis results showed marked DNA ladders pattern in the splenocyte of surgical trauma stressed rats, which can be abolished by either chemical sympathectomy or β-adrenergic receptor antagonist propranolol (Fig. 2). While neither sympathectomy nor propranolol could induce splenocyte apoptosis on normal rats.

3.3. Quantitative measurement of apoptosis by using TUNEL assay

Rats were subjected to surgical trauma stress 24 h later with sympathectomy or β-receptor antagonist propranolol. TUNEL-positive apoptotic cells in spleen sections were shown in dark brown on a methyl green counterstained background. It was found that a significant number of cells in the spleen of stressed rats were undergoing apoptosis, whereas only a few apoptotic cells were detected in the spleen of control rats (Fig. 3A). An effect of sympathectomy and propranolol on apoptosis was also observed when examined by TUNEL assay; each of the treatment will alone abolish the lymphocyte apoptosis induced by stress (Fig. 3A). To quantify the incidence of apoptosis, the number of apoptotic cells was counted and expressed as number of apoptotic cells/100 nuclei. As shown in Fig. 3B, the number of apoptotic cells was increased significantly from 1.48±0.85 in control group to 12.33±2.97 in trauma group ($P<0.001$), while pretreatment with either sympathectomy or propranolol, the

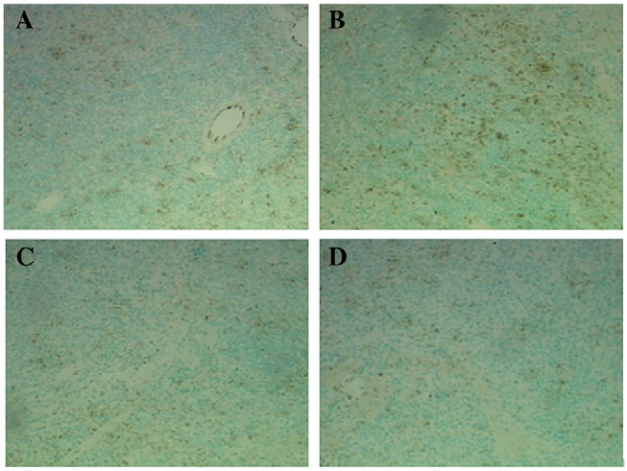

Fig. 5. Effect of surgical trauma stress on splenocyte Fas protein expression by using immunohistochemistry. A: Control; B: trauma; C: trauma + sympathectomy; D: trauma + propranolol. Photos were taken at 40×.

number of apoptotic cells was back to 2.32±0.8 in sympathectomy + trauma group; and 1.8±0.68 in propranolol + trauma group.

3.4. Fas protein expression in surgical traumatized rats

The expression of Fas protein in splenocytes was examined with or without surgical trauma stress. As shown in Figs. 4 and 5, the expression of Fas protein in splenocyte was significantly enhanced at 24 h after stress as detected by Western blot analysis. Sympathectomy as well as propranolol inhibited this stress-induced increase in Fas protein expression. The effect of stress on Fas expression was also observed when detected by immunohistochemistry.

4. Discussion

The effect of physical and psychological stress on the immune system has been noticed since the 1940s (Kiecolt-Glaser et al., 2002). Various studies with different model systems have demonstrated that stress could either enhance or reduce immune function (Ader and Cohen, 1993; Moynihan, 2003). It has been shown that stress significantly impacts leukocyte cellularity and immune response, even increase susceptibility to various diseases. Among many criteria examined upon exposure to stress, the reduction in lymphocyte mitogenic response and lymphocyte cellularity is commonly assessed. In our present experiment, we found that surgical trauma stress dramatically affected splenic cellularity, around 25–30% reduction in the number of splenocytes in stressed rat. Iwagaki and Kunes also found that stress could induce lymphopenia in surgical patients (Iwagaki et al., 2000; Kunes and Krejsek, 2000).

Sympathetic nervous system and immune system are closely correlated. Histochemical and biochemical studies demonstrated a rich noradrenergic sympathetic innervation to the bone marrow, thymus, spleen and lymph nodes. Furthermore, noradrenergic terminals have been observed directly on lymphocytes (Felten et al., 1985; Felten and Olschowka, 1987). Catecholamines released upon sympathetic nervous system activation can stimulate target cells via interacting with cell surface adrenergic receptors (Elenkov et al., 2000). There are two major classes of adrenoreceptor, α and β. Rat lymphocytes and macrophages express high-affinity β-adrenoreceptors (Abrass et al., 1985; Mackenzie et al., 1989), whereas α-adrenoreceptors have been identified only on rodent macrophages (Spengler et al., 1990) but not on lymphocytes. Stress and sympathetic activation are positively correlated. Related studies emphasized that catecholamines could suppress cellular immunity and increase the susceptibility to tumor occurrence, growth and metastasis. Stress has been shown to increase the tumor progression and the mortality in cancer patients (Li et al., 1997). To address the role of peripheral sympathetic nervous system in stress-induced splenocyte reduction in rat, sympathectomy and propranolol, specific antagonists of beta-adrenergic receptors were employed in our experiment. Administration of 6-OHDA or propranolol prior to surgical trauma completely blocked the stress-induced reduction in splenocyte numbers. The result indicated that the cell loss in rat spleen was indeed due to sympathetic nervous system stimulation. Also, the effect of propranolol indicated the involvement of β-subtype adrenoreceptor. These findings coincided with the previous reports that stress-induced immunosuppressive effects, such as inhibition of various immune cell functions in vitro, be mainly due to β-adrenergic stimulation (Exton et al., 2002; Feldman et al., 1987; Plaut, 1987).

Apoptosis is an evolutionary conserver "cell suicide" program present in all nucleated metazoan cells (Meier et al., 2000). Research in several laboratories has shown that lymphopenia after exposure to stress is in fact due to the apoptosis (Dominguez-Gerpe and Rey-Mendez, 2001; Yin et al., 1999). In our study, it was found that the cell loss was accompanied by a pronounced increase in apoptotic cells in the spleen. The enhanced rate of apoptosis was confined to the white pulp (the physiological T–B lymphocyte-rich area); red pulp areas exhibited significantly fewer apoptotic cells. The splenocytes apoptosis observed in the present study mainly was lymphocyte apoptosis. Increases in T cell apoptosis have also been observed in patients undergoing surgical procedures (Delogu et al., 2001; Oka et al., 1996). When analyzed by the TUNEL assay, 6-OHDA or propranolol was found to inhibit the appearance of TUNEL positive cells induced by surgical trauma. Therefore, the blockade of surgical trauma stress-induced splenocyte reduction and apoptosis by β-adrenergic antagonists (Fig. 1) strongly suggests a pivotal role of peripheral sympathetic system in this process. Catecholamine-mediated apoptosis of lymphocyte has been described by others (Cioca et al., 2000; Josefsson et al., 1996). We hypothesize that surgical trauma may activate the sympathetic nervous system, then the catecholamine, released from sympathetic nerve fiber, and targeted on β-receptor on lymphocyte, causing the lymphocyte to experience apoptosis.

Fas antigen is a type I membrane protein with molecular weight of 45 kDa, which belongs to the tumor necrosis factor (TNF)/nerve growth factor receptor family (Itoh et al., 1991). It is expressed on a variety of cell types including activated T and B cells, hepatocytes, and ovarian epithelial cells (Watanabe-Fukunaga et al., 1992). Fas–FasL interaction plays a critical role in the regulation of the immune system (Maher et al., 2002; Sharma et al., 2000). Loss-of-function mutation in Fas reduces apoptosis and causes the development of lymphocyte accumulation diseases in both human and mice (Lenardo et al., 1999; Wallach, 1997). In the present experiment, it was found that surgical trauma stress could increase Fas expression in splenocytes, either sympathectomy or propranolol could inhibit stress-induced Fas expression, which suggested that increased Fas protein expression might be responsible for stress-induced splenocyte apoptosis. A recent study reported that chronic restraint stress-induced lymphocyte reduction occurs through endogenous opioid mediated Fas expression, which in turn induces apoptosis (Yin et al., 1999, 2000), which provided another support for our study.

In conclusion, this study clearly demonstrated that the sympathetic nervous system especially β-adrenergic receptor was involved in surgical trauma stress-induced immune

dysfunction through an apoptotic mechanism. The increasing Fas protein expression may be the critical factor in this process. Yet, the interaction between sympathetic nervous system and other system such as hypothalamic–pituitary–adrenal axis or opioid system needs further study.

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