Original Article
Proliferation and apoptosis of Peyer’s patches and its lymphocytes in experimental terminal ileitis

Wei Wang, Ailei Xu, Guohua Zhou, Mingfang Leng, Hongyu Zhou, Jun Yan

Department of Gastroenterology, Affiliated Xiangnan Hospital of Hunan Normal University, 169 Hospital of PLA, Hengyang, P.R. China

Received October 21, 2014; Accepted December 1, 2014; Epub December 1, 2014; Published December 15, 2014

Abstract: This study will provide guide for the terminal ileitis in clinical diagnosis and treatment. The animals were been done terminal ileum-cecum side to side anastomosis, terminal ileum operation line and only anesthesia treatment, respectively. The model group presented acute inflammation after surgery for 2 weeks and the inflammation was limited to the mucosal layer. Animals presented chronic inflammation to 8 weeks, mucosal membrane was given priority to with lymphocytic infiltrates. In 2 weeks and 4 weeks, the number of Peyer’s patches (PP knot) and PP knot lymphocytes increased significantly in the model group ($P < 0.05, P < 0.01$). At 8 weeks, the suture group and the model group presented a large number of lymphocytic apoptosis ($P < 0.01$). Rat ileal PP knot lymphocyte small molecule DNA showed typical “trapezoid” bands. We observed apparent morphology of apoptosis and crescent-shaped nucleus. Continuous immune response in terminal ileitis plays a considerable role in the process of the disease.

Keywords: Cell apoptosis, lymphocytes, PP knot, terminal ileitis

Introduction
After more than 60 years, the etiology of pig proliferative ileitis has been clear joint efforts of many scientists [1]. Both acute and chronic pig ileitis is infectious intestinal disease caused by intracellular Lawson bacteria infection. The etiology and pathogenesis of human acute and chronic terminal ileitis have researched many years. Although there are a lot of progresses, in general, its etiology and pathogenesis is more complex. At present most of scholars believe that chronic terminal ileitis has relationship with infection, immune factors, colon and ileal reverse flow, allergic factors, drug factors and so on [2-4]. These researchers thought infection was initiator and immune response was the key factor. The allergic factors in the final analysis were the body’s immune response and drug factors may also be related to immune response.

In recent years, the pathogenic role of the colon and ileal reverse flow has particular concern. Whatever the reason resulting in ileum-cecum function imbalance, all can cause colon and ileal reverse flow. Then this reverse flow caused the transplantation or translocation of bacteria, the formation of endogenous infection. A series of changes mentioned above stimulated terminal ileum mucosal immune reaction. This mucosa has abundant lymphoid tissue. After mucosal immune reaction local mucosa was damaged and this harm caused terminal ileitis [5]. So that the main mechanism of chronic terminal ileitis happening may be: colon intestinal dysmotility disorders, ileocecal dysfunction, colon-ileum reflux, bacterial translocation, bacterial infection, the end of ileum mucosal inflammatory lesions. They had effect on next factor one by one.

Peyer’s knot (Peyer’s patches, PP) is small intestinal tissue of mucosa follicular. PP knot found by Swiss physician Johann Peyer and exactly was defined it as part of the lymphatic system. They located mainly in the distal small intestine mucosa lamina propria, especially in terminal ileum. PP knot is a kind of white elliptic uplift structure, slightly protruding in the small mesenteric. PP knot is aggregated by lymphoid follicles. Each PP knot contains about 5~900
lymph follicles and this number will change with age. PP knot of the experimental animals was found in the middle of pregnancy. Proliferation of lymphoid tissue happened in the embryo stage and lymphoid cell density reached to peak at birth [6]. After the birth, size and the number of PP knot increased expeditiously which showed the mucosal immune system response to antigen in the environment. This immune response occurred especially after microbial invasion happening. PP knot of germ-free animals is generally small, but it will increase contacted with microbes. If adult intestinal immune barrier is damage, bacterial translocation and intestinal infection can cause PP knot and immune cells (T lymphocytes, B lymphocytes and macrophages) apoptosis [7, 8]. We could understand terminal ileum immune response through studying PP knot and PP knot lymphocytes.

In this study, we made SD rat model for terminal ileitis to discuss the mechanism of it. We observed ileal histopathology. At the same time, we researched the number, apoptosis and proliferation of PP knot and lymphocytes in these animals. We wanted to know the changes of PP knot and lymphocytes in terminal ileitis model animals.

Materials and methods

Rats

Selected male SD rats were provided by the Ministry of Hua University and kept in a con-
Control 10 4.8±1.1 5.2±1.3 5.0±0.6 2.54±0.51 2.69±0.76 2.63±0.64
Suture group 10 5.5±0.9 7.0±1.2 5.1±1.5 3.17±0.35 3.92±1.07 2.66±0.79
Model group 10 7.1±1.4 9.4±2.6 6.3±0.7 4.01±1.12 6.45±1.36 3.11±0.93

Histology

We made the end of ileum for paraffin wax blocks. The sections (5 μm) were stained with hematoxylin and eosin (HE) [9]. We observed inflammatory cell infiltration, changes in blood vessels, lymphatic vessels, thoracic duct and intestinal villus.

Counting PP knot lymphocytes

First counting the number of PP knot from the ileocecal valve up to the end of ileum for 20 cm. PP knot were rinsed with ice-cold saline to remove blood and feces. Then we set these PP knot to a pre-cooled RPMI1640 medium (Gibco) containing 10% fetal bovine serum (Sijiqing, Hangzhou).

Detection PP knot lymphocyte apoptosis

According to the introduction of kit box, we made SD rats PP knot lymphocyte suspension first. Then we used fluorescein isothiocyanate (FITC) combined annexin-V and propidium iodide (PI) apoptosis detection kit box for cell staining. We obtained the total PP knot lymphocyte apoptosis by Flow cytometry detection.
Peyer’s patches and its lymphocytes

Table 2. Rate of SD rat PP knot lymphocyte apoptosis and proliferation in three groups (n=10, \( \bar{X} \pm s \))

<table>
<thead>
<tr>
<th>Group</th>
<th>Rat No.</th>
<th>Rate of lymphocyte apoptosis (%)</th>
<th>Rate of lymphocyte proliferation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2W</td>
<td>4W</td>
<td>8W</td>
</tr>
<tr>
<td>Control</td>
<td>1.85±0.21</td>
<td>1.91±0.28</td>
<td>1.79±0.23</td>
</tr>
<tr>
<td>Suture group</td>
<td>2.07±0.34</td>
<td>2.32±0.45</td>
<td>34.8±5.45</td>
</tr>
<tr>
<td>Model group</td>
<td>2.26±0.29</td>
<td>2.47±0.52</td>
<td>81.1±9.68</td>
</tr>
</tbody>
</table>

**PP knot lymphocyte DNA agarose gel electrophoresis**

For typical apoptotic, 200 bp nucleotide segment size and a large fold is formed in the core body. Rather than the typical apoptotic cells, the degradation of DNA in the nucleus is not entirely and form only a large 300-50 kb of DNA. With this feature, we can determine the occurrence and development of apoptosis by DNA gel electrophoresis.

According to manual operation, we used apoptotic DNA extraction kit to extract apoptotic molecule DNA. 15 g/L agarose gel electrophoresis, SYBR Green-1 staining, the gel was observed by the visible light camera and stored the results pictures.

**PP knot lymphocytes Hochst33258/PI nuclear staining**

PP node cells were collected and adjusted the cell concentration about 1×10⁶/ml. Lymphocytes were suspended in 1 mL RPMI 1640 medium (containing 10% Fetal bovine serum, FBS) then the suspension was added 10 μL Hoechst33258 (0.1 μg/ml). The mixture was incubated 37°C for 5-15 min. Then the stained cells were placed on ice and centrifuged at 4°C after cooling. Cells were resuspended by 1 mL PBS. After that we added 5 μL PI (1 μg/ml) stock solution mix into cell suspension and 4°C dark stained for 15 min. We dropped cell suspension on a glass slide and observed under a fluorescence microscope photograph.

**Statistical analysis**

Experimental results data expressed as mean ± standard deviation (± s) and the groups were compared using ANOVA (one-wayANOVA). If the difference was statistically significant, homogeneity of variance was conducted by LSD test; if not, did Dunnet’s test. SPSS17.0 was used for statistical analysis and \( P < 0.05 \) was considered statistically significant.

**Results**

**Ileal histopathology**

In control group, all tissues were normal (Figure 1A-C). In suture group, we observed capillaries, lymphatic vessels and thoracic duct dilatation after two weeks of surgery. In the mean time, tissue showed inflammatory cell infiltration, intestinal villus became thicker and shorter (Figure 1D). From Figure 1E we observed inflammation reduced significantly, epithelium and intestinal villus had integrity, inflammation cells reduced. In suture group 8 weeks after surgery, tissue had epithelial integrity and proliferation of fibroblasts, inflammatory cells reduced (Figure 1F). Model group presented acute inflammation after two weeks of the surgery performance. The inflammation was limited to the mucosa and local tissue showed mucosal neutrophil infiltration (Figure 1G). Then the inflammation gradually deepened after surgery for four weeks. Inflammatory cells infiltration became lymphocytes gradually. And congestion bleeding acute inflammation was gradually moderated, while lesions invaded the submucosa accompanied by a small amount of fibroblasts hyperplasia (Figure 1H). To eight weeks, tissue presented a typical chronic inflammation and showed mucosal lymphocyte infiltration (Figure 1I).

**Changes of SD rat PP knot number and lymphocytes**

Compared with the control group, the suture group at 2 weeks and 4 weeks, PP knot had a small increasing in the number of nodes (\( P_{2 \text{week}} > 0.05, P_{4 \text{week}} < 0.05 \)). The number of PP knot was similar with the control at 8 weeks (\( P_{8 \text{week}} > 0.05 \)) (Table 1). In model group, the number of PP knot was increased significantly at first 2 weeks and 4 weeks (\( P_{2 \text{week}} < 0.05, P_{4 \text{week}} < 0.05 \)).
0.01); at 8 weeks, PP knot had a small increase ($P_{8\text{ week}} > 0.05$) (Table 1). The changes of the lymphocytes total number in PP knot were similar with PP knot (Table 1).

Rate of SD rat PP knot lymphocyte apoptosis and proliferation

Compared with the control, there was a small number of PP knot lymphocyte apoptosis in the suture group and model group at first 2 weeks and 4 weeks. And these two groups had no significant difference ($P_{2,4\text{ weeks}} > 0.05$). At 8 weeks, the suture group and the model group had a large number of apoptotic lymphocytes ($P_{8\text{ week}} < 0.01$) (Table 2 and Figure 2). At the mean time, between the suture group and the model group had a significant difference ($P < 0.01$) (Table 2). Proliferation of lymphocytes in PP knot had a small increase at 4 weeks compared with the control ($P_{4\text{ week}} < 0.05$). There were on significant changes at 2 weeks and 8 weeks in these two groups (Table 1 and Figure 3).

Figure 2. Flow cytometry to detect cell apoptosis in three groups. A-C. Control group at 2 weeks, 4 weeks and 8 weeks. The rate of apoptosis cell was 1.85%, 1.91% and 1.79% respectively. D-F. Suture group at 2 weeks, 4 weeks and 8 weeks after surgery. The rate of apoptosis cell was 2.07%, 2.32% and 34.8% respectively. G-I. Model group at 2 weeks, 4 weeks and 8 weeks after surgery. The rate of apoptosis cell was 2.26%, 2.47% and 81.10% respectively.
SD lymphocyte apoptosis in rat PP knot characteristic DNA ladder bands

There were no small molecule 200 bp DNA bands in ileum PP knot lymphocyte DNA agarose gel electrophoresis of control group (Figure 4, 5, 6 and 7 lanes), suture group 2, 4 weeks (Figure 4, 3 and 4 lanes) and model group 2, 4 weeks (Figure 4, 1 and 2 lanes). In the suture group 8 weeks and the model group 8 weeks rat ileal PP knot lymphocyte small molecule DNA showed typical “trapezoid” (200 bp and 400 bp) bands (Figure 4, 8 and 9). The 200 bp band in model group was more evident than suture group at 8 weeks.

The staining characteristics of SD rat PP knot lymphocytes apoptosis nucleus

Compared with the control, PP knot stained lymphocytes increased significantly in the suture group and the model group at 2 weeks and 4 weeks. This result was consistent with increasing the total number of PP knot lymphocytes (Table 1 and Figure 2). At 8 weeks, compared with the control, the suture group and the model group showed a lot of lymphocyte apoptosis (Figure 5F and 5I). In the model group, we observed apparent morphology of apoptosis, nuclear condensation state and crescent-shaped nucleus (Figure 5I arrow showed).
Peyer’s patches and its lymphocytes

Discussion

Distant ileitis is mainly non-specific inflammation. Other previous studies suggested that this disease main pathogenesis: colon intestinal dysmotility disorders, ileocecal dysfunction, colon-ileum reflux, bacterial translocation, bacterial infection, the end of ileum mucosal inflammatory lesions. Typical pathological changes are: ileal mucosa and submucosa lymphoid tissue proliferation significant, lymph follicles expand and the biochemical centers significant [10, 11]. In this study, we observed that surgery induced inflammation. The suture group and the model group had acute inflammation after surgery at 2 weeks. After performing terminal ileum and cecum side to side anastomosis cecum in model group, colon bacteria continued coming into the end of the ileum. So inflammation of the model group was significantly more serious than the suture group. At 8 weeks, the suture group surgical influence disappeared, inflammation outcome, approaching epithelial integrity, normal tissue, while making module colon-ileum reflux, colon bacterial translocation infection, the end of the ileum environment changes, continue to stimulate the lymphatic system of local immune response, resulting in inflammatory damage. Experimental pathology also confirmed two weeks ileal mucosa neutrophil infiltration, four weeks of inflammatory cells into neutrophil lymphocyte infiltration gradually, eight weeks mucosal lymphocyte infiltration.

Colonic bacterial translocation after infection terminal ileum, started the innate immune response quickly [12]. Then these responses caused acute inflammation and also induced the lymphocyte-mediated adaptive immune response. Adaptive immune response is usually divided into three stages. First, the activation of antigen recognition stage, lymphocytes are stimulated and began to proliferate. Second, cell proliferation and differentiation phase, lymphocytes were stimulated by antigen, then activated and started to proliferate. In the second stage, stimulated lymphocytes differentiated into immune effector cells, in the meanwhile the body initiated apoptosis control system. Third, immune effector phase, a large number of apoptotic lymphocytes were cleared up and had proliferation rarely. Intestinal immune system is composed of a large number of immune cells and immune molecules within the intestinal mucosa and submucosa as well as mucosa-associated lymphoid tissue composition such as dispersed PP knot [13]. PP knot is assembled by lymphoid follicles enriched T cells and B cells and has a large quantities distribution in the terminal ileum. It is the major site that participate the local adaptive immune response in the gut. PP knot plays a key role in inducting and disseminating immune effector cells to the site of mucosal and these cells play immune function [14].

The mucosal immune system comprises of three basic lymphocytes components [15]. One is intraepithelial lymphocytes (IEL) distributed into a variety of mucosal epithelial cell. Two is scattered into the mucosal lamina propria lymphocytes (LPL), which contain various phenotypes of T cells and B cells. Three is Peyer’s patches (PP knot) lymphocytes. They are different in composition and effectiveness. Current research on mucosal immunity has become the hot immunology research, but few domestic reports presented. Separation and purification process of the mucosal immune system cell is more complex, which affected the extensive
mucosal immunity research. The characters and changes of intestinal mucosal lymphocytes are important mucosal immune studies. IEL and LPL separation process is relatively complicated in the three mucosal lymphocytes. IEL located in the epithelial cells and closed with them. LPL is located in the lamina propria of intestinal epithelial cells under the basement membrane. The dissociation degree of epithelial basement membrane and lamina propria will have a impact on IEL and LPL. On the one hand over dissociation of the epithelial layer may cause damage to the lamina propria, which resulted in mixing of IEL and IPL. On the other hand, epithelial cells and the basement membrane solution insufficient will induce lamina propria lymphocytes isolated by collagenase mixed with intraepithelial lymphocytes. Since PP knots were visible, they can completely oph-

thalmic cut with curved scissors. And these isolated PP knots were squeezed through the cell screening machinery. Then we got purified lymphocyte suspension. So, PP knot becomes a good choice of studying intestinal mucosal lymphocytes.

Apoptosis named program death is a life mechanism of multicellular organisms to regulate the body’s growth and maintain stability in the environment. This mechanism is controlled by genes. Program death is one of the main mode of cell aging natural death. Physiological significance of apoptosis is to ensure normal growth and development, maintain homeostasis, plays an active defense [16]. So apoptosis is a normal physiological process. In fact, too much or too little apoptosis can cause diseases. Similarly, we can also detect apoptosis to react

Figure 5. The staining characteristics of SD rat PP knot lymphocytes apoptosis nucleus (Bars =20 μm). A-C. Control group at 2 weeks, 4 weeks and 8 weeks. D-F. Suture group at 2 weeks, 4 weeks and 8 weeks after surgery. G-I. Model group at 2 weeks, 4 weeks and 8 weeks after surgery. At 8 weeks, compared with the control, the suture group and the model group showed a lot of lymphocyte apoptosis (F and I). In the model group, we observed apparent morphology of apoptosis, nuclear condensation state and crescent-shaped nucleus (arrow showed).
Peyer's patches and its lymphocytes

the development of disease. Prominent feature of apoptotic cells is nuclear DNA fragmentation due to activation of the endogenous endonuclease. Under light microscope, apoptosis showed nuclear chromatin condensation, nucleolus fragmentation, a small bubble-like membrane formed inside the membrane. In fact, the membrane structure is still intact. The apoptotic cells eventually can be divided into several parcels of apoptotic bodies. The biochemical changes of cell apoptosis are: DNA fragment degraded regularly, DNA breakage happen between nucleosome. These degradation and breakage resulted fragment containing a different quantity nucleosome units. During agarose gel electrophoresis, small molecule DNA showed typical “trapezoid” (200 bp and 400 bp) bands. Its size is an integer multiple of 180-200 bp. If it is typical apoptosis, 200 bp nucleotide segment and a large number of size time’s segments are formed in the nucleus. With this feature, you can determine the occurrence and development of apoptosis by DNA gel electrophoresis.

In this experiment, 2, 4 and 8 weeks after surgery, suture group and model group were in the acute inflammatory phase (innate immune phase and antigen recognition activation stage), cell proliferation and differentiation phase, immune effector phase. Four weeks after surgery, in the suture group the number of PP knot and PP knot total lymphocyte count have increased compared with the control group; the rate of apoptosis increase was not obvious. In the model group, within the colon bacterial translocation and infection were observed; PP knot number and PP knot total lymphocyte count were significantly higher than the suture group and the control group; a slightly higher rate of lymphocyte apoptosis was showed. The activated lymphocytes played immune effector and then a large number of them had apoptosis after surgery 8 weeks. At this period, the number of PP knot and PP knot lymphocyte in experiment group (suture group and model group) decreased compared to 2 and 4 weeks surgery. Lymphocytes nuclear staining showed apoptotic nuclear morphology and typical “trapezoid” bands presented in DNA agarose gel electrophoresis. In the model group, colonic bacteria translocated to terminal ileum continuously. This translocation stimulated the immune response in intestinal lymphatic system. Lymphocyte apoptosis in the model group was significantly higher than the suture group, at the meantime PP knot and PP knot lymphocyte number slightly more than that in the suture group and the control group. In the pathogenesis of experimental terminal ileitis, SD rats experienced innate immunity and adaptive immunity stages. PP knot and PP knot lymphocyte presented significant proliferation first and apoptosis in the later time changes. Thus, we sustained that immune response played an essential role in its pathogenesis.

Numerous studies demonstrated that lymphocytes, especially intestinal intraepithelial lymphocytes play an important role in the pathogenesis of terminal ileitis. Intestinal intraepithelial lymphocytes mainly transferred to the junction between the epithelium from the lamina propria lymphocytes or PP knot lymphocytes. The changes number and activity of intestinal intraepithelial lymphocytes and PP knot lymphocytes were still not very clear. Local immune response of the terminal ileum mainly cellular immunity or humoral immunity is also not understood. How the relationship between immune response, nervous and endocrine relationship? Since this experiment only studied the changes in PP knot and PP knot lymphocytes, those areas above mentioned were not involved. So we will study specific circumstances in further.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Guohua Zhou, Department of Gastroenterology, Xiangnan Hospital of Hunan Normal University, 169 Hospital of PLA, Hengyang 421002, P.R. China. Tel: +86138-75662956; E-mail: guohuazhou01@163.com

References


Peyer’s patches and its lymphocytes


