Effects of seaweed extract on Vβ13 gene expression and Treg cells in virus-induced type 1 diabetes mellitus

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Abstract: Objective To measure the expression of Vβ13 mRNA and Treg cells in spleen lymphocytes from rats infected with cytomegalovirus (CMV) as well as Kilham rat virus (KRV) and the therapeutic effects of seaweed extract on type 1 diabetes mellitus. Methods Forty LEW, 1WR1 rats were randomly divided into two groups. The rat model of type 1 diabetes mellitus was established using rat cytomegalovirus combined with KRV. The rats in the treatment group were treated with seaweed extract daily for 40 days, while the experimental group was given the same amount of sterile saline. The expression of Vβ13 mRNA in the spleen was assessed by RT-PCR and CD4⁺ CD25⁺ Treg cells were detected by flow cytometry (FCM). The sections in splenic cells were detected by HE staining. Results In the experimental group, 90% of the rats were diabetic (18/20), and the mean blood glucose level was 3.15 g·L⁻¹. The expression of Vβ13 in the experimental group was higher than that in the treatment group. The ratio of CD25⁺ Treg cells in the experimental group was 6.2% and in the treatment group 13.5% (P<0.05). Conclusion The expression of Vβ13 mRNA was significantly higher in the early stage of virus-induced diabetes, and the proportion of CD4⁺ CD25⁺ Treg cells was lower in the experimental group. Seaweed extract increased the Treg cell expression for the treatment of diabetes.

Key words: cytomegalovirus; type 1 diabetes; seaweed extract; Vβ13; Treg cells

海藻提取物对病毒感染的同化力 1 型糖尿病 Vβ13 基因表达及 Treg 细胞的影响

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摘要: 目的 观察 Vβ13 mRNA 和 Treg 细胞在大鼠细小病毒(KRV)联合巨细胞病毒(CMV)感染大鼠的脾淋巴细胞中的表达以及海藻提取物对 1 型糖尿病的治疗效果。方法 LEW, 1WR1 大鼠 40 只, 随机分成 2 组, 应用 CMV 联合 KRV 建立大鼠 1 型糖尿病模型。治疗组大鼠每日口服海藻提取物共 40 d; 成模组用等量无菌生理盐水替代同步进行。RT-PCR 检测脾脏 Vβ13 mRNA 的表达, 流式细胞术检测脾细胞 Vβ13⁺ CD4⁺

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CD25^+ Treg 细胞亚群比例；HE 染色观察糖尿病胰岛的病变情况。结果 实验组结果为 90% (18/20)，成模血糖平均水平在 3.15 g/L，实验组 Vβ13 的表达高于治疗组，实验组中 CD4^+ CD25^+ Treg 细胞的比例为 6.2%，治疗组为 13.5% (P<0.05)。结论 感染乙型肝炎病毒的大鼠 Vβ13 mRNA 的表达在糖尿病初期显著增高。Vβ13^+ CD4^+ CD25^+ Treg 细胞亚群比例较低。海藻提取物提高了 LEW.1WRI 大鼠 Treg 细胞的表达，可用于治疗糖尿病。

关键词：乙型肝炎病毒；1 型糖尿病；海藻提取物；Vβ13；Treg 细胞

Type 1 diabetes is an autoimmune disease in which T cell-mediated islet beta cells are selectively disrupted. An epidemiological survey has shown that cytomegalovirus infection and diabetes are closely related. Our preliminary results confirmed that cytomegalovirus (CMV) combined with Kilham rat virus (KRV) leads to diabetes, while blocking Vβ13 expression effectively prevents and treats type 1 diabetes. Recent studies have shown that the variable region (V region) Vβ13 gene located in the TCRβ chain is an important susceptibility gene for type 1 diabetes. Our preliminary study also confirmed that LEW.1WRI pre-vaccinated Vβ13 antibody, can block and inhibit the progress of diabetes in rats. However, the change of Vβ13 gene expression in T cells and the changes of Vβ13^+ regulatory T (Treg) cells in the pathogenesis of viral diabetes mellitus are still not clear.

In this study, Vβ13 and CD4^+ CD25^+ Treg expression were assessed by establishing a rat model of type 1 diabetes mellitus induced by virus infection, so as to explore the effect of seaweed extract on Vβ13 in the early stage of the disease.

1 Materials and methods

1.1 Reagents

Anti-CD4-PE and anti-CD25-FITC were from BD, San Jose, CA, USA. Sequences coding for Vβ13 were from GenBank, and RT-PCR primers were designed with Primer-BLAST online software. The primers are listed in Table 1 and were synthesized by Shanghai Sangon Biotech Co.

### Table 1 Primers used for PCR

<table>
<thead>
<tr>
<th>Genetic</th>
<th>Element</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vβ13(F)</td>
<td>339 bp</td>
<td>5’-ATGGGCGACGCGTCTTGTCC-3’</td>
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<tr>
<td>Vβ13(R)</td>
<td>5’-</td>
<td>5’-ACTGCTGGCACAGAGAT-3’</td>
</tr>
<tr>
<td>GAPDH(F)</td>
<td>143 bp</td>
<td>5’-GCACCACCAACTGTTCAGCAC-3’</td>
</tr>
<tr>
<td>GAPDH(F)</td>
<td>5’-</td>
<td>5’-GCACCGCCGATAGGCAG-5’</td>
</tr>
</tbody>
</table>

1.2 Viruses, animals, and model

Forty normal LEW, 1WRI rats kindly provided by Professor Mordes of the University of Massachusetts Medical School were kept in Weifang Medical College Animal Experimental Center. Rat cytomegalovirus (RCMV) and Kilham rat virus (KRV) were gifts from Dr. Bruggeman of Maastricht University, Denmark, and Professor Mordes. The experimental group was the diabetic model group. Diabetes modeling was performed according to a previous study: 1 × 10^6 PFU of RCMV in 1 mL, and 1 × 10^6 PFU of inducer KRV in 4 × 1 mL were injected intraperitoneally. The day of injection was defined as the first day. The control group was injected with normal saline. Ten days later, blood glucose was
measured by the method of streptozotocin-induced diabetic nephropathy (3 times/week). Diabetes mellitus was diagnosed when the blood glucose level was \(>2.50 \text{ g/L}\) for 2 days. The rats were randomly divided into 2 groups, 20 rats in each. Blood glucose was measured in both groups for 40 days. Rats were sacrificed after anesthesia at the end of the experiment. The pancreas and spleen tissue were rapidly removed and preserved in liquid nitrogen until use.

1.3 Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of Weifang Medical University (No. 2016-012). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

1.4 Histological Observations

The sections were stained with paracrine-eosin (HE) and observed under a light microscope. The pancreatic tissues were embedded in paraffin. After 24 h, the tissues were fixed in 80% ethanol. The number of pancreatic islets in each section was counted.

1.5 RT-PCR was used to assess \(\beta\)13 gene expression in spleen cells

Total RNA from each group was extracted and reverse-transcribed into cDNA for PCR detection of mRNA expression changes of \(\beta\)13 in cells from each group. Products were separated by electrophoresis on 1.5% agarose gel and bands were visualized and analyzed using ImageJ (NIH) to transfer gray scale into numerical values. Product/GAPDH values were calculated for statistics.

1.6 Detection of T cell subsets

Pre-chilled PBS (10 mL) was added to a 10 cm Petri dish. Rats were sacrificed and immersed in 70% ethanol for 1 min. Through a sterile laparotomy, the spleen was removed and placed in the dish. A spleen cell suspension was prepared by conventional methods, a sample (\(10^{-3}\) L) was added into the flow cytometer tube, and 10 \(\mu\)L anti-rat CD4 and CD25 labeled with different fluoresceins were added. The cells were kept in an ice bath for 30 min, and after washing twice with cold PBS, the ratio of CD4\(^+\) cells and CD4\(^+\)CD25\(^+\) cells in the spleen were measured by flow cytometry.

1.7 Statistical analysis

All data were analyzed using SPSS 13.0 statistical software. Values are expressed as mean±standard deviation. The percentages of CD4\(^+\)CD25\(^+\) cells were analyzed using one-way ANOVA and a difference was considered statistically significant when \(P<0.05\).

2 Results

2.1 Type 1 diabetes model caused by virus infection

Rats were inoculated with RCMV and KRV virus (Fig. 1). In the experimental group, the incidence of diabetes was 90% (18/20), while in treatment group, only 10% (2/20) showed symptoms (\(P = 0.0003\)). As in the previous study, diabetes usually occurred in the experimental group in the 15～20 days after inoculation.
2.2 Pathological changes in the pancreas

In the treatment group, the number of pancreatic islets was evenly distributed, and the islets were mostly round or elliptical, scattered among the pancreatic acini. The islets were clear, without structural change or lymphocyte infiltration. In the experimental group, the islets showed vacuolar degeneration or were empty. The islets were atrophied, the number of islets is reduced, their distribution sparse, their number reduced. In addition, there were varying degrees of pancreatic changes and different degrees of lymphocyte infiltration (Fig. 2).

2.3 Vβ13 expression in splenetic cells

The expression of Vβ13 mRNA in the spleen of rats in the experimental group was increased, but was lower in the treated and the control groups (Fig. 3).

Note: A. Representative RT-PCR of Vβ13 mRNA expression in spleen cells from the experimental and treatment groups. Lanes 1~3: experimental, treatment, and control groups; B. Relative mRNA levels of Vβ13 mRNA analyzed by RT-PCR (GAPDH served as internal control). *P<0.01 versus experimental group

2.4 Percentages of CD4⁺ CD25⁺ Treg cells in splenocytes

Compared to the treatment group, the Treg cells from spleens in the experimental group decreased after 7 days, when the CD4⁺ CD25⁺ Treg rate was 6.2%, while in the treatment group, it was 13.5% (P<0.05).
3 Discussion

Type 1 diabetes is a T-lymphocyte-mediated autoimmune disease characterized by immune islet inflammation and selective islet beta cell damage. The incidence of type 1 diabetes is closely associated with cytomegalovirus infection. This infection may induce autoimmune injury of islet beta cells in individuals with genetically susceptible genes. So far, the pathogenesis of this disease has not been clear\textsuperscript{[8]}. The incidence of type 1 diabetes has a certain genetic predisposition, but may also be induced by virus infection and other environmental factors. In recent years, different groups, including ours, have used infection with different strains of virus to generate diabetes mellitus models, and have confirmed that different viruses affect the progress of type 1 diabetes. Among these, CMV infection is general in the Chinese population, the overall infection rate reaching 70% to 100%\textsuperscript{[9\textsuperscript{-}10]}, and increasing evidence links CMV infection with type 1 diabetes. KRV was first isolated from tumor-bearing rats by Kilham et al. (1959) and belongs to the genus Parvovirus. Both experimental and wild-type rats are natural hosts of KRV. Adult rats infected with KRV are asymptomatic, but immune suppression and other factors can stimulate type 1 diabetes. Other reports and this study have shown that KRV induces a better experimental infection model of type 1 diabetes. In preliminary work, we showed that rat CMV combined with KRV causes type 1 diabetes in rats, but its Vβ13 mRNA and CD4\textsuperscript{+} CD25\textsuperscript{+} Treg cells had not been reported.

The role of the T cell receptor (TCR) in the pathogenesis of type 1 diabetes mellitus is an active area of research. Recent studies have shown that the variable region (V region) Vβ13 gene located in the TCR\β chain is an important susceptibility gene for type 1 diabetes\textsuperscript{[k]}. Our preliminary study also confirmed that vaccination with Vβ13 antibody blocks and inhibits the progress of diabetes in LEW.1WR1 rats\textsuperscript{[l]}.

Based on their participation in the pathogen-
esis of type 1 diabetes. T cell surface markers can be divided into CD4\(^+\) and CD8\(^+\) T cell subsets. Among these, the CD4\(^+\) subset of Th1 are involved in cell-mediated immunity, and the Th2 cell sub-group in humoral immunity. Normally, Th1/Th2 maintain a dynamic balance\(^{[11-12]}\). In the present study, a large number of islets and beta cells disappeared in the control group, while CD8\(^+\) T cells were predominant in the control group. In the experimental group, only one mouse had islet inflammation, and no CD8\(^+\) T cells were found, suggesting that oral insulin pathways significantly inhibit CD8\(^+\) T cells (possibly cytotoxic CD8\(^+\) T cells) localized in the islets, thus protecting the islets and beta cells, CD4\(^+\) CD25\(^+\) regulatory T cells, which are recently-discovered subsets, are important regulators of immune stabilization and inhibit the activation and proliferation of CD4\(^+\) and CD25\(^+\) T cells, B cells, and NK cells after activation. Treg cell dysfunction leads to immune imbalance and the proliferation of inflammatory T cells in vivo. The transcription factor Foxp3 is specifically expressed in Treg cells and is a characteristic expression factor.

Seaweeds are rich in dietary fibres, unsaturated fatty acids, and polyphenolic compounds. Many of seaweed compounds have been reported to be beneficial to health including in managing diabetes. Currently, there is a growing awareness of the role of food beyond basic nutritional value by providing health benefits and reducing the risk of various illnesses including diabetes\(^{[13,14]}\). Seaweeds are rich in bioactive compounds in the form of polyphenols, carotenoids, vitamins, phycobilins, phycocyanins, and polysaccharides, and many of these are known to have beneficial applications in human health. Therefore, the potential of various beneficial components in seaweeds and their possible modes of action against the development of diabetes deserve closer investigation.

In summary, in this experiment, we used virus infection in LEW. IWR1 rats to investigate the effect of seaweed extract on Vβ13 expression. The expression of Vβ13 mRNA in spleen T cells and the changes of CD4\(^+\) CD25\(^+\) T cell subsets were determined by RT-PCR and flow cytometry to further establish the important role of Vβ13 in type 1 diabetes mellitus. We found that seaweed extract significantly reduced Vβ13 expression, and increased the number of Treg cells to improve the diabetes. Our further research will focus on the possible mechanisms by which seaweed extracts reduce diabetes, including the down-regulation of Vβ13.

References


