Effect of Rosuvastatin on Regulatory T Cell in Atherosclerosis of ApoE KO Mice*

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ABSTRACT Objective: To investigate the effect of Rosuvastatin on regulatory T cell in atherosclerosis of Apoe KO mice. Methods: 30 atherosclerotic models of ApoE KO mice were established, then were randomly divided into hypercholesterolemia diet group (control group), low doses of Rosuvastatin treatment group and high dose group. Each group was interfered with distilled water or Rosuvastatin for 8 weeks, and the aortic roots of the animals were harvested. The atherosclerotic plaque size was detected by oil red staining and the expression of regulatory T cell in atherosclerotic plaque was detected by immunohistochemistry. Results: There were the formation of atherosclerotic plaque in each group of mice; However, the atherosclerotic plaque size in low dose group and high dose group were obvious smaller than that in the untreated mice ($P<0.01$). Rosuvastatin increased significantly the expression of Treg, which was dose-effect relationship. Conclusion: Rosuvastatin had the effect of promoting the production of regulatory T cell to inhibit atherosclerosis.

Key words: Atherosclerosis; Rosuvastatin; Regulatory T cell; ApoE KO mice

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Introduction

Atherosclerosis (AS) is an immune inflammatory disease [1]. In addition to regulating blood lipids, many studies have shown that statins which have become the first line of prevention and treatment of AS and its complications, have anti-inflammatory and immunomodulatory functions. Regulatory T cells (Treg) are a group of immune suppression of T cell subsets, of which different subtypes have different cell surface markers [2], and in which $CD4^+$ $CD25^+$ Treg is present more in-depth study of Treg. A large number of researches have found that $CD4^+$ $CD25^+$ Treg played an important role in inhibiting the progress of atherosclerosis. Various molecules expressed in the cell surface, but were not the specific markers. It has been proved [3] that Foxp3, of which mRNA and code Scurfy (referred to as Sf) expressed particularly in $CD4^+$ $CD25^+$ Treg, was recognized as the specific marker of $CD4^+$ $CD25^+$ Treg. The atherosclerotic formation of apolipoprotein E knockout (ApoE KO) mice is very similar to human, which used to be studied on the pathogenesis of atherosclerosis [4]. With the establishment of atherosclerotic model through feeding ApoE KO mice on high cholesterol diet, and by the semi-quantitative detection of Foxp3 in atherosclerotic plaques with immunohistochemistry (sp) to reflect the expression of regulatory T cells, this study will detect the changes of Treg in atherosclerosis of ApoE KO mice with Rosuvastatin treatment, and discuss the correlation between statins and Treg, which can provide new prospect for the application of traditional anti-AS drugs.

1 Materials and methods

1.1 Materials

Drugs and experimental animals: Rosuvastatin was provided by AstraZeneca. Apolipoprotein E gene knockout (Apolipoprotein E-knockout, ApoE-KO) mice were 30, 8 weeks old, SPF grade, male and female, weighing 18-22 grams, strain C57BL/6J, and purchased from Chinese Academy of Medical Experiments Institute of Zoology, with animal quality certification SCXK (Beijing) 2005-0013.

1.2 Methods

(1) Preparation of high-fat diet composition: 10 % lard, 2.5 % cholesterol, sodium cholate, 66.5 % mice standard diet.

(2) The establishment of atherosclerotic model and grouping management: After the introduction, the mice were bred with free food intake and drinking water in the animal breeding room of SPF level, given high fat diet after adaptive feeding for 1 week, and fed continuously for 12 weeks; Then the mice were randomly divided into 3 groups: The control group continued to be given high fat diet, with normal saline 0.2 ml orally, 1 time/day (Other groups were so); On this basis, the low dose group and high dose group were respectively given 2.5 mg/kg and 5.0 mg/kg of Rosuvastatin, 1 time/day. The dose was adjusted according to the weight per week, consecutively for 8 weeks.

(3) Specimen handing: The animals were anesthetized with 10 % chloral hydrate for the taking of their hearts and blood vessels, which were then washed in PBS.

(4) Assessment of plaque area in aortic root: From aortic root to the end of abdominal aorta, the aorta was amputated and fixed in 10 % formalin for 24 hours. The aortic root was cut out for a

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continuous line of frozen sections, in which each slice thickness was 10 μm and each mouse contained about 40 sections covering about 400 μm. After taken one every 100 μm and five slices each mouse, the slices were pasted in the polylysine-treated slides to spare. The slides were stained with oil red O, before the counter staining with hematoxylin. After the image acquisition by Pathological microscope camera system, Densitograph Imaging software was used to analyze the plaque area of each section.

(5)Immunohistochemical staining scores: After fixed with 4% paraformaldehyde, the remaining aorta tissues were embeded in paraffin and sliced into 5 μm of thickness, which were then attached to the polylysine-treated glass slides; After being kept for 10 minutes at room temperature respectively with 1 % methanol hydrogen peroxide and antigen retrieval solution, and blocked for 20 minutes at room temperature with normal goat serum, the sections were incubated overnight at 4 ℃ with primary antibodies(dilution, 1:100 for Foxp3), and then incubated for 20 min at 37 ℃ with biotinylated secondary antibodies (IgG) and streptavidin-horseradish avidin HRP working solution(SA/HRP), respectively. After stained with DAB solution, staining with hematoxylin, thorough dehydration and mounted with neutral gum, the sections were detected by optical microscope. The brown particles which could be seen in the atherosclerotic plaques were regarded as positive staining, as well as the nuclei were stained blue. The aforementioned methods were used for immunohistochemical staining to evaluate Foxp3 expression in the atherosclerotic plaques with staining integral. By the imaging systems, the staining intensity of all the positive areas was graded: Negative staining was 0, weak positive staining scored one mark, positive staining scored two marks and strong positive staining scored three marks and the positive cells seen within the plaques were classified in 5: Positive cells accounting for 0 to 1 % of all the cells scored 0, 1 % to 10 % scored 1 points, 10 % to 50 % scored 2 points, 50 % to 80 % scored 3 points, 80 % to 100 % scored 4 points. Immunohistochemical score (IHS) of each observed area was the product of the two.

(6) Statistical Methods: SPSS 16.0 software was used for statistical analysis. Measurement data were expressed as mean± standard deviation ($\bar{x}\pm s$). Differences between the groups were analyzed by one-way ANOVA. $P<0.05$ was considered as statistically significant.

2 Results

2.1 Assessment of atheromatous plaque area

The result of oil red staining showed that there were clear formations of atherosclerotic plaques in mice of each group; However, the atherosclerotic plaque size in Rosuvastatin-treatment group, including low dose group and high dose group, were smaller than that in the untreated mice ($P<0.01$), and the difference between the control group and high dose group was more remarkable. (Figure 1 and Table 1).

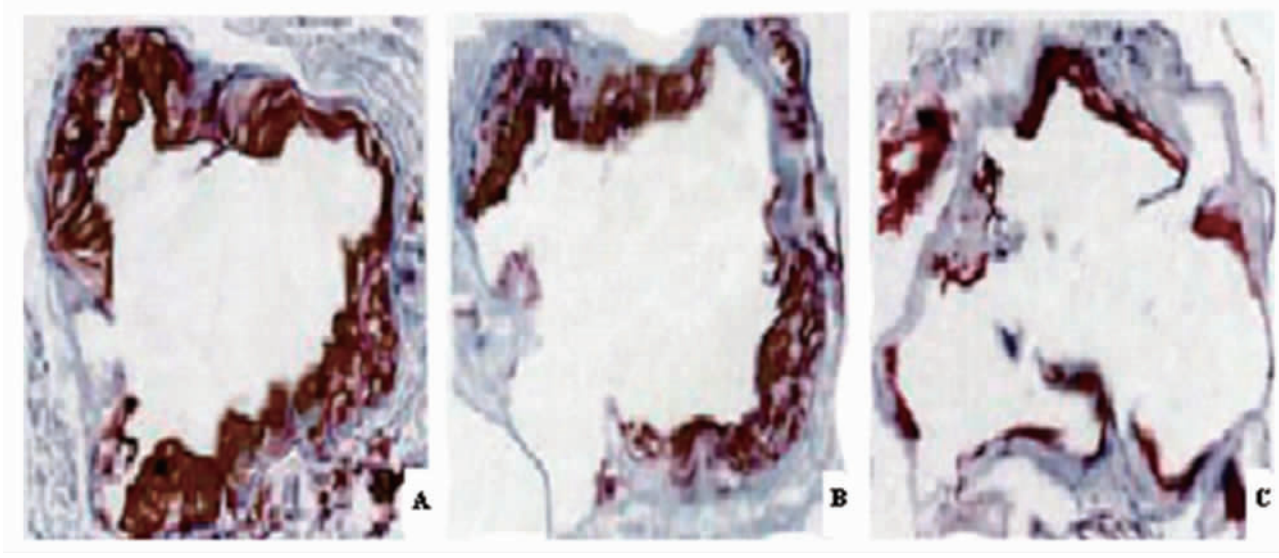

Fig. 1 Atherosclerotic lesion in each group of ApoE KO mice

A :Control group; B :Low dose group; C :High dose group

2.2 The expression of Foxp3 in each group

The expressions of regulatory T cells were determined by the expressions of Foxp3 in each group, which were analyzed by immunohistochemistry. There was positive expression of Foxp3 and the positive cells in each group (Figure 2),. Compared with that of the control group, Foxp3 protein in low dose group and high dose group expressed in a higher level. Image analysis system was used to evaluate the immunohistochemical integral of Foxp3 (Table 2). It could be found that, the expressions of Foxp3 in low dose group and high dose group were higher than that in the control group ($P<0.01$), which showed dose-effect relationship.

Table1 Comparison of atherosclerotic lesion in aortic root of ApoE KO mice

Groups	Atherosclerotic plaque area (mm^2)	Atherosclerotic plaque area / luminal area (%)
Control group	0. 23 ± 0.04	37.7 ± 4.3
Low dose group	0.16 ± 0.05[1)]	25.3 ± 6.5[1)]
High dose group	0.05 ± 0.02[2)]	8.4 ± 1.3[2)]

Note: Compared with Control group, 1)P<0.01; Compared with Low dose group, 2)P<0.01

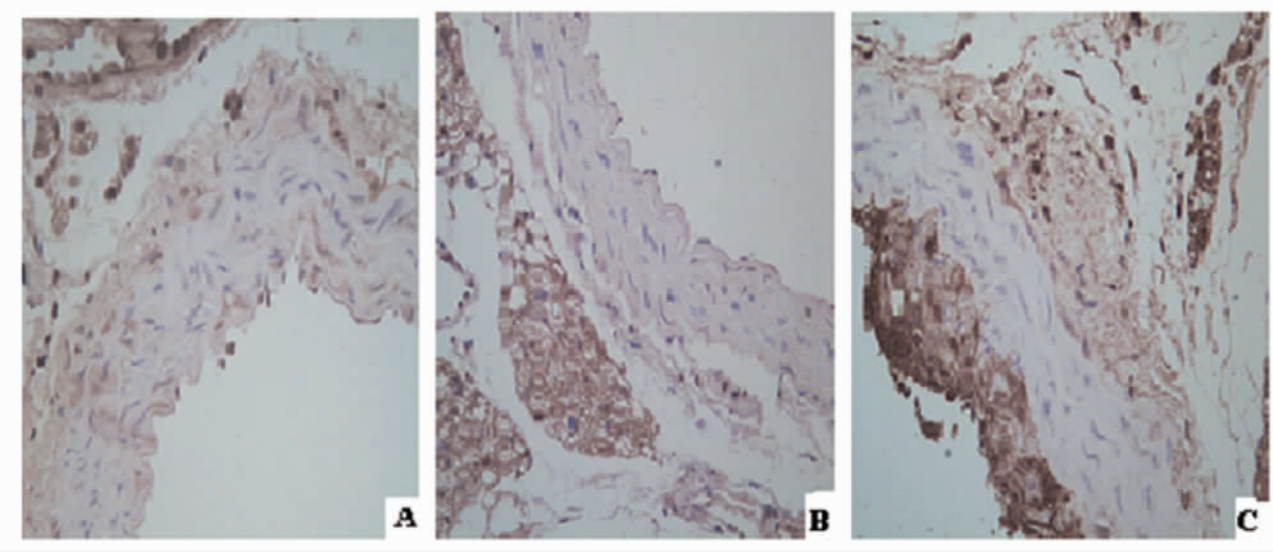

Fig.2 Foxp3 expression in aortic plaque

A :Control group; B :Low dose group; C :High dose group

3 Discussion

The pathological processes included endothelial injury, the expression of adhesion molecule, monocyte moving into the subcutaneous part, the adhesion and migration of leukocyte, the oxidized low density lipoprotein uptake, the formation of foam cell, the release of cytokines，as well as the migration and proliferation of the smooth muscle cells, finally form atheromatous plaques. Now it is widely recognized that immunological mechanisms play an vital role in the process of AS [5]. Activated T cells exist in each stage of atherosclerotic lesions and T cell-mediated immune response contributes to the progress of AS. $CD4^+$ T cells account for the majority of T cells in atherosclerotic plaques of human and animal models, among which the Th1, Th2 subsets accelerate the activation of macrophages and inflammation by secreting IFN-γ , IL-2, TNF-α , etc, and thereby show the effect that promote the progress of atherosclerotic lesions and the loss of plaque stability[6]. Regulatory T cells are a group of immune suppression of T cell subsets, with the regulation of autogeneic response as the main function. Mallat Z had pointed out that, a new subtype of T cells，$CD4^+$ $CD25^+$ Treg had control over the occurrence and development of AS, which could restrain the initial T cell proliferation by the secretion of cytokines (such as IL-10 and TGF-β) or direct interaction between the cells and reduce the pathological response of Th1 and Th2, showing the resistance to atherosclerosis [7,8]. Ait-Oufella H verified the effect of $CD4^+$ $CD25^+$ Treg on atherosclerosis in following ways [9]: 1) Atherosclerosis induced by the injection of cd28-/ - spleen cells into Apoe-/- Rag2-/- mice could be eliminated by the injection of $CD4^+$ $CD25^+$ Treg. 2)T cells and macrophages within atherosclerotic lesions decreased after the injection of $CD4^+$ $CD25^+$ Treg. These studies confirmed that Treg was closely related to atherosclerosis, which could be inhibited by the maintenance of the number and function of Treg. The new view is that the formation and development of atherosclerotic plaque is caused by an imbalance between pathogenic Th1 cells and (or) Th2 and Treg [10].

It is well-known that statins have been proved to be the only one in the overall lipid drugs to reduce cardiovascular and cerebrovascular disease. Besides reducing blood fat, improving endothelial function, inhibiting smooth muscle cell proliferation and stabilizing plaques, statins also have anti-inflammatory and immunomodulatory functions. By treating the mice sufferred from atherosclerosis with rosuvastatin, compared with the mice without the treatment of Rosuvastatin, this study indicated that not only the plaque area in the former was significantly reduced, but also regulatory T cells were more expressed (P<0.01), which was speculated that it might promote the production of regulatory T cells and its secreted inhibitor of inflammation to restrain atherosclerosis; Furthermore, with the increasing of dose, the up-regulation of regulatory T cells was more obvious, but the optimum dose need to

be confirmed by subsequent researches.

As a way statins treat atherosclerosis, regulatory T cells will likely provide new targets and open up new ideas for prophylaxis and cure of AS. However, the research of atherosclerotic immune mechanisms and the discovery of Treg were recently proposed, and therefore the immune regulation methods with Treg as a target for treatment of AS is still a relatively new research direction. Owing to few studies in this area, the specific changes of Treg in each period of atherosclerosis currently remain unclear. In two clinical trials from abroad, with immunological tests on coronary plaque in patients sufferred from stable angina, unstable angina pectoris (UAP) and acute myocardial infarction, it was found that with the increase of plaque instability, the ratio of $CD4^+$ $CD25^+$ Treg in total T cells grew gradually , while some researches have shown that Treg in peripheral blood decrease in ACS [11,12]; Moreover, the specific mechanisms of statins action on regulatory T cells are not yet clear, which need further research and exploration. According to several recent studies, the maturation of dendritic cells plays an important role in T cell differentiation [13]; Inhibiting the maturation of dendritic cell may be one of the mechanisms of atorvastatin action on Treg, because immature dendritic cells can induce the differentiation of Treg [14].

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瑞舒伐他汀对载脂蛋白 E 基因敲除小鼠动脉粥样硬化中调节性 T 细胞的影响 *

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摘要 **目的**:探讨瑞舒伐他汀对载脂蛋白 E 基因敲除(ApoE KO)小鼠动脉粥样硬化中调节性 T 细胞的影响。**方法**:首先将 30 只 ApoE KO 小鼠建立动脉粥样硬化模型,随机分为高胆固醇饮食组(对照组)、瑞舒伐他汀低剂量组和瑞舒伐他汀高剂量组,各组分别给予蒸馏水或瑞舒伐他汀进行干预 8 周;将主动脉根部行冰冻切片油红染色,评估粥样硬化斑块面积大小;免疫组织化学法检测主动脉根部粥样硬化斑块处调节性 T 细胞(Treg)的表达。**结果**:各组小鼠均有动脉粥样硬化斑块形成,采用瑞舒伐他汀治疗的小鼠动脉粥样硬化斑块的面积明显小于未经治疗的小鼠($P<0.01$),同时瑞舒伐他汀能明显增加粥样硬化病变处调节性 T 细胞的表达,且呈现剂效关系。**结论**:本实验观察到瑞舒伐他汀不仅能减小 ApoE KO 小鼠的主动脉粥样硬化斑块,且能使调节性 T 细胞的表达增多,推测瑞舒伐他汀可以通过促进调节性 T 细胞的生成而起到抑制动脉粥样硬化的作用。

关键词:动脉粥样硬化;瑞舒伐他汀;调节性 T 细胞;ApoE KO 小鼠

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