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Effect of pioglitazone combined with simvastatin on the CD40-CD40 ligand system in rabbits with atherosclerosis

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Abstract. – OBJECTIVE: This paper aims to investigate the interaction mechanism between pioglitazone/simvastatin and the CD40-CD40 ligand (CD40-CD40L) system and to determine their interaction effects on atherosclerosis in rabbits.

MATERIALS AND METHODS: Forty rabbits were randomly divided into five groups of eight: normal control, hyperlipidemia model, pioglitazone, simvastatin, and pioglitazone combined with simvastatin therapy. The rabbits were raised for 16 weeks. Blood samples and the aortic length were taken after 16 weeks with the following indicators: (1) blood lipid measurement [total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C)] were measured; (2) measurement of serum high-sensitivity C-reactive protein (hsCRP), soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble intercellular adhesion molecule-1 (sICAM-1), soluble CD40 ligand (sCD40L), and matrix metallopeptidase-9 (MMP-9) by enzyme-linked immunosorbent assay; (3) aortic pathological observation and measurement of the area ratios for plaque/intimal; and (4) expression determination of CD40L in plaque parts by immunohistochemistry.

RESULTS: In the treatment groups, the levels of TC, TG, LDL-C, hsCRP, sVCAM-1, sICAM-1, sCD40L, and MMP-9 increased, and HDL-C level, plaque/intimal area ratio, and CD40 expression in the plaque parts decreased. Improved effects were also found in the combination treatment group.

CONCLUSIONS: Pioglitazone and simvastatin may inhibit different functions, such as inflammatory response and lipid regulation, by inhibiting the CD40-CD40L signaling pathway to suppress the formation of atherosclerosis. Therefore, the combined application of pioglitazone and simvastatin has synergistic effects.

Key Words:
Atherosclerosis, Pioglitazone, Simvastatin, CD40-CD40 ligand.

Introduction

Cardiovascular diseases caused by atherosclerosis (AS) are the leading cause of death today, making AS one of the most popular research topics. Epidemiological, clinical, and laboratory studies on AS at the population, whole organ, cellular, molecular, and even genomic and functional genomic levels[1,2] revealed that AS is a multifactorial chronic inflammatory disease[3]. The CD40-CD40 ligand (CD40-CD40L) system located upstream of the cytokine network is an important signaling pathway in the inflammatory process[4]. The CD40-CD40L system is crucial in T and B lymphocyte activation and humoral-mediated immunity. CD40 and its ligand CD40L are commonly found in various cells and platelets associated with AS[5]. They can regulate various inflammatory responses and are thought to be a key link in inflammation in AS[6]. Numerous anti-AS treatments directly or indirectly act through this pathway. Therefore, mechanisms by which the CD40-CD40L signaling pathway can be inhibited to prevent AS have increasingly attracted the attention of researchers.

Some scholars found that antidiabetic drugs, such as thiazolidinediones (TZDs), have an anti-AS effect by improving insulin resistance and lowering blood sugar[7]. However, the specific mechanism is not fully understood. Statins are currently the most powerful and effective lipid-lowering drugs. These statins also have lipid-lowering effects, such as improving endothelial functions and inhibiting inflammatory reactions, thereby inhibiting the occurrence and development of AS. In this study, the influence of pioglitazone combined with simvastatin on the forma-

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tion of AS in rabbits and the CD40-CD40L signaling system was observed. The functions and potential mechanisms of pioglitazone combined with simvastatin in inhibiting the genesis and development of AS were also discussed to provide a theoretical basis for clinical applications.

Materials and Methods

Experimental Animals

Forty healthy male Japanese white rabbits (age: 3 to 4 months) weighing (2.0 + 0.15) kg were provided by the Experimental Animal Center of Liaoning Medical University. 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 animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Third Affiliated Hospital of Liaoning Medical College.

Model Preparation

The 40 experimental rabbits were raised in single cages. The duration of the adaptive feeding was 2 weeks. The rabbits were randomly divided into five groups of eight; the control group (A) with ordinary pellets, the hyperlipidemia model group (B) with a high-fat diet (provided by the Cholesterol Beijing Bonding Biopharmaceutical Co., Ltd.), the pioglitazone group (C) with a high-fat diet and 0.5 mg/(kg·d) pioglitazone (GlaxoSmithKline Co., Ltd.) gavage, the simvastatin group (D) with a high-fat diet and 2.5 mg/(kg·d) simvastatin (Zhejiang Jingxin Pharmaceutical Co., Ltd.) gavage, and the combination group (E) with a high-fat diet, 0.5 mg/(kg.d) rosiglitazone, and 2.5 mg/(kg.d) simvastatin gavage. The duration of the entire experiment was 16 weeks.

Observational Indexes and Method Measurement

The vein blood from the rabbit ears was collected after a fasting period of 12 h before the experiment and at the end of week 16. (1) Total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) were detected using an automatic biochemical analyzer. (2) The serum specimens were prepared after the experiment, and high-sensitivity C-reactive protein (hsCRP), soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble intercellular adhesion molecule-1 (sICAM-1), soluble CD40 ligand (sCD40L), and matrix metallopeptidase-9 (MMP-9) were measured by enzyme-linked immunosorbent assay (Beijing Biosynthesis Biotechnology Co., Ltd., Wuhan Boster). The animals were killed through air embolism. The aorta was removed under aseptic conditions, placed in 10% formalin, and then fixed. The specimens with the most evident AS plaques were embedded in paraffin to prepare hematoxylin and eosin staining pathological and immunohistochemical pathological sections. (3) Oil red O staining was performed to measure the plaque/intimal area ratios. (4) Immunohistochemistry was conducted to determine the expression of CD40 on the plaque sites.

Statistical Analysis

Data were analyzed using SPSS13.0 statistical software (SPSS Inc., Chicago, IL, USA). All data were presented as mean ± standard deviation (± s). Two groups were compared using q test. Statistical significance was considered at $p < 0.05$. Images and a comparative description method were used to perform pathological and immunohistochemical comparisons.

Results

TC, TG, LDL-C, and HDL-C Measurements

Except for group A, all groups showed significant differences in the levels of TC, TG, LDL-C, and HDL-C before and after the 16-week experimental period ($p < 0.01$). Compared with group A, groups B, C, D, and E had increased levels of TC, TG, and LDL-C levels but decreased level of HDL-C ($p < 0.01$). Compared with group B, groups C, D, and E had significantly lower ($p < 0.01$) levels of TC, TG, and LDL-C but significantly higher ($p < 0.01$) level of HDL-C. Compared with groups C and D, group D had lower levels of TC, TG, and LDL-C ($p < 0.05$) but higher level of HDL-C ($p < 0.05$) (Table I).

hsCRP, sVCAM-1, sICAM-1, sCD40l, and MMP-9 Measurements

After 16 weeks, the hsCRP, sVCAM-1, sICAM-1, sCD40l, and MMP-9 levels of groups B, C, D, and E were significantly higher ($p < 0.01$) than those of group A. The hsCRP, sVCAM-1, sICAM-1, sCD40l, and MMP-9 levels of groups C, D, and E were significantly lower ($p < 0.01$) than those of group B. The hsCRP, sV-

Table I. Changes of TC, TG, LDL-C and HDL-C (mmol/L) in rabbit serum before and after experiment among groups ($\bar{x} \pm s$).

Groups	TC	TG	LDL-C	HDL-C
Group A	1.35 + 0.25	0.87 + 0.21	0.71+ 0.11	0.88 + 0.12
Group B	25.81 + 11.32*	2.87 + 1.22*	14.53 + 9.82*	1.3 + 1.5*
Group C	19.54 + 5.5*,#	1.02 + 1.83*,#	7.3 + 2.85*,#	2.7 + 1.2*,#
Group D	20.32 + 10.8*,#	2.42 + 1.33*,#	6.89 + 2.47*,#	3.4 + 1.4*,#
Group E	16.22 + 4.3*,#,◊	0.99 + 0.91*,#,◊	4.97+2.12*,#,◊	2.1 + 0.9*,#,◊

Note: Compared with Group A: $^{**}p < 0.05$, $^{*}p < 0.01$. Compared with Group B: $^{\#\#}p < 0.05$, $^{\#}p < 0.01$. Compared with Group C and D: $^{\lozenge}p < 0.05$.

Table II. Test results of serum hsCRP (mg/L), sVCAM-1, sICAM-1, sCD40L, MMP-9 (ng/L) indicators ($\bar{x} \pm s$).

Groups	hsCRP	sVCAM-1	sICAM-1	sCD40L	MMP-9
Group A	0.407 + 0.304	1.23 + 0.26	3.42 + 4.33	0.49 + 0.13	11.15 + 1.79
Group B	1.532 + 0.183*	5.32 + 0.48*	10.54 + 23.75*	6.28 + 1.65*	41.32 + 2.98*
Group C	1.052 + 0.145*,#	3.05 + 0.93*,#	6.45 + 14.72*,#	3.98 + 0.44*,#	31.29 + 2.34*,#
Group D	1.210 + 0.152*,#	3.36 + 0.82*,#	7.74 + 13.69*,#	4.41 + 0.82*,#	35.27 + 2.01*,#
Group E	0.982 + 0.129*,#,◊	2.91 + 0.87*,#,◊	5.75 + 11.28*,#,◊	3.50 + 0.77*,#,◊	27.44 + 1.99*,#,◊

Note: Compared with Group A: $^{**}p < 0.05$, $^{*}p < 0.01$. Compared with Group B: $^{\#\#}p < 0.05$, $^{\#}p < 0.01$. Compared with Group C and D: $^{\lozenge}p < 0.05$.

CAM-1, sICAM-1, sCD40l, and MMP-9 levels of group E were significantly lower ($p < 0.05$) than those of groups C and D (Table II).

Pathological Morphological Observation and Plaque/Intimal Area Ratios

Light microscopy revealed that the intima of group A was smooth, thin, and continuous and that the endothelial and smooth muscle cells were neatly arranged. The localized intima of group B significantly thickened with numerous plaques. Some porridge neoplasia formed on the vessel walls. The lipid core and a large number of foam cells were observed in the plaques, which were infiltrated by macrophages and lymphocytes. The medial smooth muscle cells were disordered. Groups C and D had generally less and thin plaques than the model group.

Oil Red O staining revealed that the treatment groups had lower plaque/intimal area ratios than group A. Compared with groups C and D, group E had significantly lower plaque/intimal area ratios ($p < 0.05$; Table III, Figure 1).

Immunohistochemistry

The expression of CD40 in the aortic plaques was detected in groups B, C, D, and E. Compared with group B, groups C, D, and E had significantly decreased expression levels of CD40 in the aortic plaques ($p < 0.01$); compared with groups C and D, group E had decreased expression level of CD40 ($p < 0.05$) (Table III, Figure 2).

Table III. Plaque/intimal area ratios and the percentage of immunohistochemical positive area ($\bar{x} \pm s$).

Groups	Plaque/intimal area ratios	CD40	CD40L
Group A	0 + 0	95.23 + 7.15	110.49 + 7.84
Group B	0.52 + 0.132	52.78 + 6.33#	49.07 + 7.42#
Group C	0.344 + 0.115‡	77.35 + 4.09#,‡	63.89 + 3.72#,‡
Group D	0.304 + 0.126‡	70.86 + 4.76#,‡	57.41 + 4.87#,‡
Group E	0.251 + 0.094‡,◊,*	82.27 + 4.43#,‡,◊,*	70.35 + 4.21#,‡,◊,*

Note: Compared with Group A: $^{\#}p < 0.01$, Compared with Group B: $^{\ddagger}p < 0.01$. Compared with Group C: $^{\lozenge}p < 0.05$. Compared with Group D, $^{*}p < 0.05$.

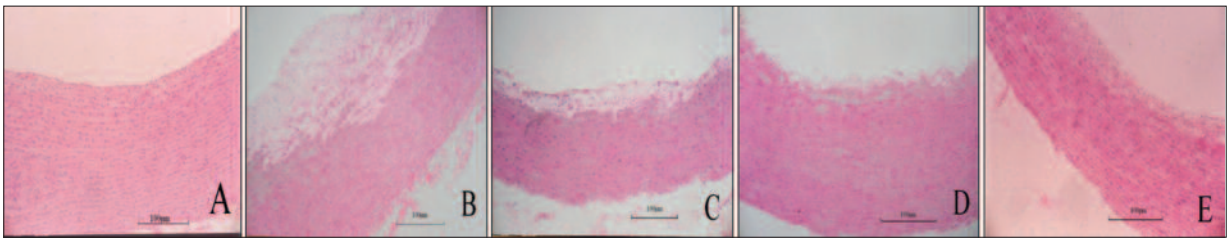

Figure 1. Comparison of HE staining for aortic intima among different groups (×100). ***Group A*** (the normal control group): aortic endothelial cells were tight, smooth muscle cells lined well, and no foam cells deposited in the subendothelial. ***Group B*** (the hyperlipidemia model group): aortic intima showed diffuse bulge and large amounts of foam cells were seen. ***Group C*** (the pioglitazone group): medium thickness fat spots and a moderate amount of foam cells were seen in aortic intima. ***Group D*** (the simvastatin group): thin grease spots and a small amount of foam cells were seen in aortic intima. ***Group E*** (the combination therapy group): only scattered limited grease spots formation were seen in aortic intima and the number of foam cells was significantly less than that of the group D.

Discussion

Understanding AS requires a long and arduous process of exploration. Experts that focused on understanding AS have reached a consensus: AS is a pathological change that can be affected by numerous factors[8]. The main risk factors include LDL elevation, HDL reduction, high blood pressure, diabetes mellitus and obesity, metabolic syndrome, and systemic inflammation. Previous studies confirmed that CD40-CD40L is commonly found in the arterial plaques of various cells, such as macrophages, smooth muscle cells, and T cells, in the process of AS. The CD40-CD40L system significantly affects cell functions that are associated with AS. This system is also closely related to the genesis and development of plaques. The distribution of CD40-CD40L is consistent with the distribution of functional molecules, such as adhesion molecules, cytokines, matrix metalloproteinases, and tissue factors, which are closely related to the genesis and development of AS[11]. Previous *in vivo* and *in vitro* studies confirmed that personal, local, or exogenous CD40L can stimulate the expression of AS plaque-associated cells, such as lymphocytes, endothelial cells, smooth muscle cells, and macrophages, and generate a series of active materials related to plaque genesis, plaque rupture, and thrombus formation[12]. Some scholars believed that CD40L is a potential marker for AS plaque instability[13]. The new pathway regulating the expression of CD40-CD40L provides a new therapeutic target for AS. In the present study, the levels of serum hsCRP, serum vascular adhesion molecules, soluble intercellular adhesion molecules, sCD40L, and MMPp-9 significantly decreased in the drug-treated groups. Immunohistochemistry results showed that the expression levels of CD40 in the AS plaques of the drug-treated groups were lower than those of the model group. This result indicates that pioglitazone and simvastatin have anti-AS functions and are related to the blocking of the CD40-CD40L signaling pathway.

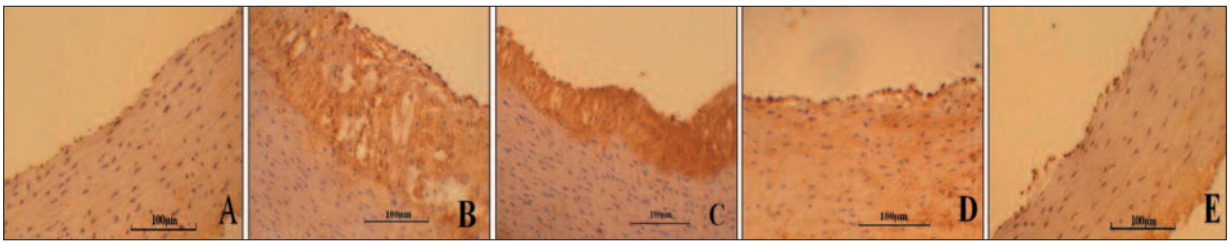

Figure 2. Comparison of CD40 expression based on the immunohistochemical results for aortic intima among different groups (×100). ***Group A*** (the normal control group): no specific staining in aortic intima. ***Group B*** (the hyperlipidemia model group): specific staining for aortic intima was apparent strongly positive, a lot of brown particles were seen. ***Group C*** (the pioglitazone group): amounts of brown particles were seen in aortic intimal plaques, specific staining was positive. ***Group D*** (the simvastatin group): specific staining for aortic intima was positive. ***Group E*** (the combination therapy group): the brown particles in atherosclerotic plaques were positive staining granules, and a small amount of brown particles were seen.

Pioglitazone is one of the PPAR-γ agonists. After activation, PPAR-γ inhibits the generation of superoxide anion and the expression of redox-sensitive transcription factor[14] (which inhibits pro-inflammatory cytokines). In addition, inflammatory cytokines target gene activation and transcription. As a result, the expression levels of the pro-inflammatory cytokines interferon-γ, tumor necrosis factor-α, and interleukin-2 are reduced[15]. Interferon-γ, tumor necrosis factor-α, interleukin-2, and other cytokines can increase the expression of CD40-CD40L mRNA to increase the expression of CD40 on the surface of macrophages and activated T cell[16]. Therefore, the activation of PPAR-γ indirectly reduces the expression of CD40-CD40L[17] to create a protective effect in AS. These findings also suggest that pioglitazone Ming PPAR-γ inhibits the expression of CD40-CD40L in the development of AS to inhibit the formation of AS plaques.

Statins are inhibitors of hydroxymethyl glutaryl coenzyme A, which regulates blood lipids by inhibiting cholesterol synthesis. Previous studies showed that statins can improve endothelial function[18], inhibit vascular inflammation[19], and inhibit the development of porridge plaques to stabilize the plaque[20]. The current work showed that simvastatin can reduce the expression of CD40-CD40L; however, the mechanism requires further exploration. We combined pioglitazone with simvastatin to explore whether different drug combinations are effective in inhibiting the occurrence and development of AS. We also determined whether the various drug combinations can reduce the side effects caused by large doses of a single drug to overcome the drawbacks of decreased efficacy with increasing dose. Compared with the single-drug group, the drug combination therapy group had lower plaque area and significantly improved blood lipids, inflammation, and other functional indicators. Experimental results suggested that pioglitazone combined with simvastatin can interfere with the occurrence and development of AS by blocking the CD40-CD40L system. Therefore, pioglitazone and simvastatin showed a synergistic effect in inhibiting the occurrence and development of AS.

Conclusions

This experiment provided a new target for the clinical treatment of AS and expanded the clinical applications of pioglitazone to innovate the prevention and treatment of AS. Given the short period for the animal experimentation in this study, the side effects of long-term application require further investigation. The next step is to perform long-term clinical trials to clarify the interaction effects of the two types of drugs.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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