The Effection of All-trans Retinoic Acid (ATRA) on the Expression of MCP-1 and TLR-4 in Atherosclerotic Lesions of Rabbits

REN Na, DONG Guo-xiong△, ZHANG She-hua, ZHANG Bin, HU Zhen-zhen

(Department of Cardiology, the Affiliated Hospital of Qingdao University Medical College, Qingdao, Shandong 266003, P.R.China)

ABSTRACT Objective: To investigate the efficacy of ATRA on the tunica intima proliferation, the expression of MCP-1(monocyte chemotatic protein-1), TLR-4 (Toll-like receptor4) in the injury and repair lesion located at the intima of the rabbit carotid artery, in order to study the possible mechanism of anti-inflammation of ATRA. Methods: New Zealand male rabbits were randomly divided into 9 groups (n=6) control group (ABC) experimental group (ABC), sham operated group (ABC). Except the sham operated group, the other two groups had been given high fat diet for two weeks. And the rabbits of control and experimental group were given air drying operation to injure the carotid artery intima. In the sham operated group, carotid arteries were dissected and exposed without injuring the intima. The rabbits of experimental group were not given ATRA intragastric administration three days before the operation until the execution. The rabbits of group A were put to death 7 days after the operation, group B 14 days, and group C 28 days. Then the specimens of carotid arteries were took the and the morphology of atherosclerotic lesions were detected and measured At last, the expression levels of MCP-1 and TLR-4 were detected by the method of immunohistochemisty. Results: ①In the control group, the intima started to proliferate 7 days after the air seasoning operation. After 14 /28 days the intima proliferated obviously with lumen stenosis, atheromatous plaque on the vessel wall and much expression of MCP-1 and TLR-4; ②In the experimental group, the intima proliferated much less severely. 14 and 28 days after the operation, the area of the proliferating intima was smaller ($P<0.05$), the opaque was thinner ($P<0.05$), and the positive expression of MCP-1 and TLR-4 was smaller ($P<0.05$) than those of the control group; ③ In the sham operated group, the intima didn't proliferate, MCP-1 and TLR-4 had little expression. Conclusions: ATRA alleviated the intima proliferation and lumen stenosis after the carotid artery intima injured by inhibiting the expression of MCP, TLR-4 and the other inflammatory factors probably.

Key words: ATRA; Inflammation; MCP-1; TLR-4

Chinese Library Classification(CLC): R541.4 Document code: A

Article ID:1673-6273(2012)05-823-06

Introduction

Atherosclerotic(AS) heart disease has become one of the diseases that threaten the health and life of human beings. As a result, the diagnosis, the prevention, the treatment and the prognosis have turned into a very important subjects which people focus on. In recently researches on the pathogenesis of the disease, it is found that many kinds of inflammatory factors help the formation of AS lesions. So researching the molecular mechanism and pathological changes of inflammatory processes, studying the preventive and therapeutic effects of drugs on inflammatory reactions may become the new target points of anti-atherosclerotic treatment. In the past researches, it was found that ATRA could inhibit the proliferation of smooth muscle cells by suppressing the signal transduction pathway of vascular smooth muscle cells proliferation[1-2]. Recently, some researches indicated that ATRA could inhibit AS inflammatory reactions by restraining the expression of vascular inflammatory factors[3]. With the progression of the research in AS mechanism, it is discovered MCP-1and TLR-4 helped the formation of atherosclerosis. By building rabbit model of carotid artery intima injury, the article is aimed at observing the effects of ATRA on inhibiting the intima proliferation after the intima injury and on the expression of MCP-1and TLR-4 in atherosclerotic lesion, studying the mechanism of alleviating intima proliferation by inhibiting inflammatory reactions, and further supplying theoretical bases of applying ATRA as a new kind medicine in the inhibiting AS.

Author:Ren Na, (1982-), Female, Master, Mninly angaged in Atherosclerotic Lesions Tel:15063097536, E-mail: maggie915@126.com

△Corresponding author: Dong Guo-xiong(1956-), male, Master, Chief Physician, Professor, Master Instructor. E-mail:qydgx@public.qd.sd.cn

(Received:2011-07-16 Accepted:2011-08-10)

1 Material and Methods

1.1 Animal grouping and model building

54 healthy New Zealand male rabbits (Institute of Animal Science and Veterinary Medicine Shandong Academy of Agricultural Sciences, License No. SCXK(Lu) 20040013, mass2.3± 0.14 kg) were randomly divided into 9 groups (n=6) :control group (ABC), experimental group (ABC), sham operated group (ABC). In the control and experimental groups, the rabbits took 150g high fat forage per day (6% hog lard 1.5%cholesterol 92.5% basic forage), and the rabbits of the sham operated group were feed with 150 g basic forage. All the three groups drank water freely. After feeding them for 2 weeks, the rabbits of control and experimental groups were given the air seasoning operation. The rabbits of experimental group were given ATRA (Shandong liangfu pharmaceutical co., ltd, 6 mg /(kg·d), dissolved in 1ml vegetable oils) intragastric administration three days before the operation to the execution. The

other dealings were the same with the control groups. In the sham operated group, carotid arteries were dissected and exposed without injuring the intima. 400 thousand units penicillin was given intramuscular per day until the third day after the operation. The rabbits of A were put to death 7 days after the operation, B 14 days, and C 28 days. Then the lesion region was divided into 4~5 parts equally, segments were fixed in 10% neural buffered formaldehyde solution and embedded in paraffin. Finally, we made transverse sectional slices of the vessel specimen (4μm).

1.2 The vessel morphology measurement and atherosclerotic lesion detection

The vessel segments were firstly observed in naked eyes after removing the vessel segments. The paraffin slices were dealt with HE staining. Then structures were observed in microscope and did image analysis with Image-Pro Plus image analysis system. Afterwards, sectional intima area (IA) and media area (MA) were measured. The mean value of IA and MA was drawn. 14 and 28 days after the operation respectively, the vertical distance from the internal elastic membrane to the cavosurface where the intima lesion of the carotid artery was the thickest were measured. We viewed the distance as the greatest opaque thickness.

1.3 MCP-1and TLR-4 expression detection

MCP-1and TLR-4 were dealt with immunohistochemisty staining with the SP two- stage method. The dilution of primary antibody (Wu Han Boster Company) was 1 :100. And was used as secondary antibody and adopted DAB coloration and hematoxylin counterstaining. MCP-1 was positive if the cytoplasm was buffy or brown. TLR-4 was positive if the cell membrane was buffy or brown. PBS substituted the primary antibody as negative control. Image analysis was did with IPP. Then positive area percentage and the average optical of positive staining in 6 high power fields per slice were measured. Afterwards, the average value was drawn. The positive expression index was the product of multiplication of the two averages multiplied by 100.

1.4 Statistical Analysis

All data were processed with the statistical software SPSS17.0. Measurement data were represented with ($\bar{X}\pm$ S). We applied Stuent-Newman-Keuls method between groups. P<0.05 was considered statistically significant.

2 Results

2.1 Macroscopic observation

All animals were disease-free and alive observing by naked eye. After the operation, the wound was not polluted with well healing. In the sham operated group, the tunica intima was smooth. In the control group, the vessel wall was stiff. Greasy spots and fatty steaks appeared on the intima in A. In B, opaque formed and the intima was unsmooth. And in C, the intima thickened and the opaque varied in length and protruded out of the intima. In the experimental group, greasy spots and fatty steaks could be seen on the intima in A. In B and C, the extent of the lesion was less severely than that in control group.

2.2 Observation by Light Microscope

In the sham operated group, the structures of intima were complete and continuous. ①Each layer arranged regularly and stuck tightly to internal elastic membrane. The structures of internal elastic membrane were complete. The tunica media was uniform in thickness with well-distributed matrix. All smooth muscle cells arranged in ring-form with spindle-shaped nuclei orienting with one accord. ② In control A, the intima proliferated in the form of simple endothelial cell mostly with stratified cells locally. The internal elastic membrane was vague with many fractures. Many smooth muscular cells went through the internal elastic membrane. In the media close to the internal elastic membrane, the extracellular matrix increased and the cells arranged irregularly. In control B, the intima proliferated more severely than that in A with much atheromatous opaque. The internal elastic membrane was damaged more heavily and cells of tunica media arranged more irregularly. Many smooth muscular cells accumulated under the endothelia with inflammatory cells infiltration in the intima. In control C, the lumen surface was uneven with much more obvious intimal roliferation. The injury and repair opaque protruded out of the intima. There were large quantities of foam cells, lipid ponds and lipid cores with much heavy inflammatory cells infiltration. The smooth muscular cell proliferated and transmigrated obviously. In the media, it appeared smooth muscular cells transferring into foam cells. ③ Compared with that in the control group, the extent of intamal proliferation, atheromatous opaque formation, the damage of internal elastic memebrane, inflammatory cells infiltration, foamy changes of cells and smooth muscular cell proliferation and transmigration were less severely in the experimental group.

2.3 Image Analysis

IA and MA was measured to represent the proliferating extent of the intima and media. 7 days after the operation, comparing IA and MA between the control and experimental group, it was statistically insignificant (t =1.8485, 0.8706 P >0.05) .14 and 28 days after the operation, in control group, the intima proliferated obviously with arranged neointima irregularly.The cross section area of the lumen decreased significantly. The IA values of experimental group were smaller than those of concurrent control group. It was statistically significant (Table1 t=16.398, 16.274 P<0.05). There was statistically insignificant for MA value in the three groups at the same period (P>0.05) .

The measurement of the thickness of the injury and repair opaque: 14 days after the operation, the thickest opaque in the control group was in 0.14± 0.05 mm, and which was the experime-

ntal group. The thickness of opaque in the experimental group was smaller than that in the control group, and it was statistically significant (t=4.597, P <0.05). 28 days after the operation, the thickest opaque in the control group was in 0.23± 0.04 mm, the experimental group was in 0.17 ± 0.04mm. It was statistically significant (t=3.621, P <0.05).

Table 1 IA and MA of three groups ($\bar{x}\pm s$)

Groups	Case numbers	IA (mm)	MA (mm^2)
A group of sham operated group	6		0.34± 0.04
B group of sham operated group	6		0.34± 0.04
C group of sham operated group	6		0.34± 0.05
A group of control group	6	0.07± 0.01	0.38± 0.06
B group of control group	6	0.23± 0.02	0.35± 0.05
C group of control group	6	0.34± 0.02	0.35± 0.05
A group of experimental group	6	0.06± 0.01	0.34± 0.05
B group of experimental group	6	0.12± 0.01●	0.34± 0.05
C group of experimental group	6	0.18± 0.02●●	0.35± 0.04

P.S. In comparison with the B control group, ● t=16.398, P<0.05; in comparison with the C control group, ●● t=16.274, P<0.05

2.4 The results of Immunohistochmisty

In the sham operated group, MCP-1 expressed little in the endothelia. 7 days after the operation, there was much MCP-1 expressing in the cytoplasm of tunica intima cells and tunica media cells in the control group, especially neoeintima cells.14 and 28 days after the operation, in the control group, the expression of MCP-1 increased. On the endothelial cells, smooth muscular cells, foamy cells and MCP-1 expressed in large quantity. In the experimental group, the regularity of MCP-1 expression was the same with that of the control group, but the positive expression index was lower than that in control group. In the sham operated group, there was no expression of TLR-4. 7days after the operation, in the control group, TLR-4 expressed a little in the intima, and more on the membrane of cells in the media. 14 and 28 days after the operation, in the control group, the expression of TLR-4 increased. In the endothelial cells and smooth muscular cells, TLR-4 expressed in large quantity, especially the smooth muscle of media. In the experimental group, the regularity of MCP-1 expression was the same with that of the control group, but the positive expression index was lower than that of control group(Table2).

Table 2 The comparison of positive expression index between control group and experimental group ($\bar{x}\pm s$)

Groups	Case numbers	MCP-1	TLR-4
A group of sham operated group	6	2.30± 0.07	2.20± 0.72
B group of sham operated group	6	3.89± 0.16	4.34± 0.66
C group of sham operated group	6	3.69± 0.22	2.79± 0.17
A group of control group	6	0.95± 0.12◆	0.92± 0.28◆◆
B group of control group	6	2.37± 0.15▲	2.33± 0.54▲▲
C group of experimental group	6	2.11± 0.12■	2.07± 0.06■■

P.S.: The comparison of MCP-1 and TLR-4 of A,B and C groups between the control and experimental group respectively, ◆ t=23.890, P<0.05; ◆◆ t=13.944, P<0.05; ▲ t=17.255, P<0.05; ▲▲ t=16.315, P<0.05; ■ t=15.573, P<0.05; ■■ t=10.030, P<0.05.

3 Discussions

In 1999, Ross R pointed definitely that AS was a kind of inflammatory disease[4]. In recent years, more and more researches proved the expression of many kinds of inflammatory factors was related to the formation of AS. MCP-1 is one member of chemotactic factors CC subfamily, whose gene is located at 17th chromosome. Its cDNA has a 5' untranslated region in the length of 53nt and a 3' untranslated region in 53nt. Its open reading frame encodes a polypeptide chain containing 99 amino acid residues, of which the last 76 amino acid residues are the mature MCP-1 and the first 23 amino acids that are hydrophobic which are the part of signal peptide. Many researches show that the chemotactism of monocyte is mainly realized by MCP-1 [5]. MCP-1 expressed in many

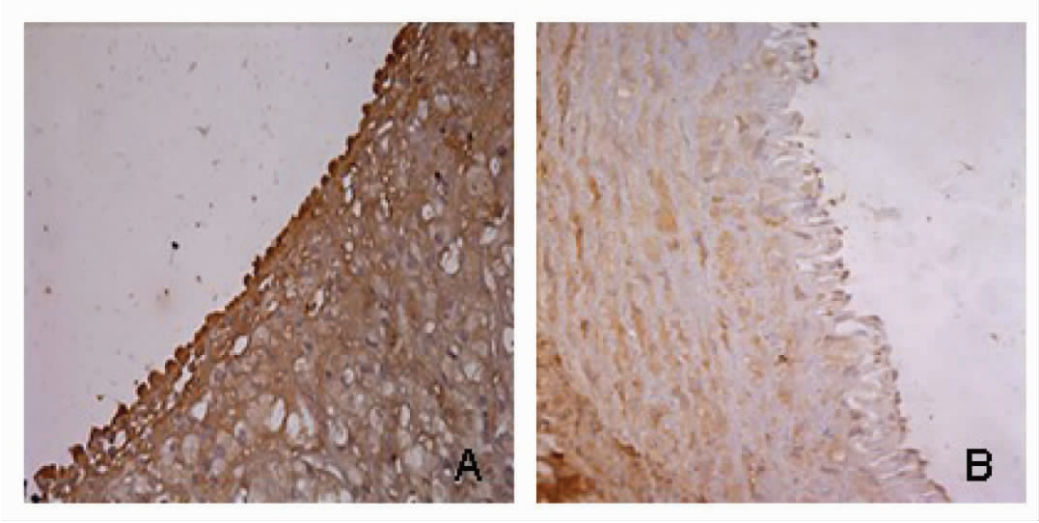

Fig.1 Expression of MCP-1 in carotid artery of rabbits in the 28th day after surgery (400)
A: Control group; B: Treatment group

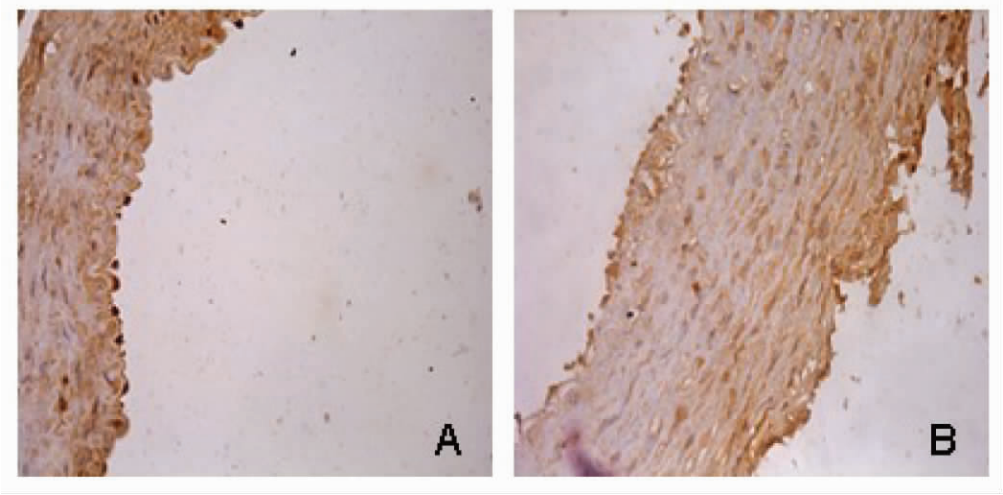

Fig.2 Expression of TLR-4 in carotid artery of rabbits in the 28th day after surgery (400)
A: Control group; B: Treatment group

kinds of cells, especially the three main cells constituting AS opaque, including monocyte/ macrophage, endothelial cells and smooth muscle cells, which are participating the formation of AS opaque directly or indirectly. In recent years, it is researched that immunological reactions run through the whole process of AS. Toll-like receptors, especially TLR-4, as one kind of transmembrane signal transduction receptors of mediating nature immunological reactions, are drawing more and more attention in the process of AS. Toll-like receptors belong to type I transmembrane protein. Its extracellular structures which are made of repetitive sequences rich in leucine participate the recognition of ligand. Its intracellular structures, called TIR region(TLR/IL-R1), which are similar to the intracellular region of interlukin-1 receptor1(IL-1R1) which are responsible for activated signal transduction. More and more evidences indicated that TLR-4 participates the process of the onset and progression of AS and the rupture of opaque and it could help the development of AS by multi-pathway, which has been recorded in details in domestic references [7]. In conclusion, MCP-1 and TLR-4, as two important representatives of inflammatory factors, play a role that cannot be ignored in the formation of AS.

The research explored the rabbit model of carotid artery injury and repair, simulating the carotid artery stenosis led by atheosclerotic opaque formation. This study observed the effects of ATRA short-term interference on the intimal proliferation and the expression of inflammatory factors of the rabbit carotid artery that was dealt with air drying operation. The experiment showed that in microscope there was a lot of inflammatory cells infiltration in control and experimental group, which suggested inflammation may play an important role in the formation of atherosclerotic opaque. The comparison between the two groups indicated that ATRA could relieve the extent of intima thickening, smooth the degree of proliferation and transmigration of muscle cells, the damage of internal elastic membrane and the infiltration of inflammatory cells. Comparing control and experimental group, the expression of MCP-1 and TLR-4 differed significantly, which indicated that ATRA inhibited the progression of inflammatory reactions, thus relieving inti-

ma proliferation and the formation of atherosclerotic opaque probably by inhibiting the expression of the two inflammatory factors.

ATRA, a natural derivative of vitamin A, plays a role in inhibiting cell proliferation and inducing normal cell differentiation. Clinically, it has been widely used to treat malignant tumors, dermatosis and acute promyelocytic leukemia. Our previous work has proved that ATRA can block the transformation, migration and proliferation of smooth muscle cells, inhibit proto-oncogene and suppress secreting extracellular matrix to form excessive proliferating intima. Whether in vivo or in vitro, ATRA can inhibit the proliferation of vascular smooth muscle cells [8-14], decrease the proliferation of intima, and then prevent AS. Some research proves that ATRA can inhibit the expression of cytochrome P450 aromatase[15] and suppress the formation of AS after the balloon injury of rabbit carotid artery [16]. At the present time, domestic researches show ATRA prevents inflammatory cells infiltration and slows down the development of nephritic syndrome by inhibiting the expression of MCP-1[17]. Now there are few reports about that ATRA inhibits the progression of inflammation by inhibiting the expression of TLR-4. This research shows that ATRA plays a part in anti-atherosclerosis by inhibiting the expression of TLR-4.

In conclusion, ATRA inhibits the intima proliferation after the intima injury, thus inhibits the progression of AS. ATRA inhibits the progression of inflammatory processes probably by suppressing the expression of MCP-1, TLR-4 and many other inflammatory factors. But given the progression of AS is a complicated process whose pathogenesis involves many inflammatory factors and signal pathway, the mechanism of ATRA inhibiting inflammatory reactions needs to be studied further.

References

[1] Zhang SH, Wang YN, Guo LL, et al. The effect of All-trans retinoic acid on VAMC proliferation signal transduction pathway in atherosclerotic lesion of the rabbit carotid artery [J]. Molecular Cardiology of China, 2007, 7(1): 30-33(In Chinese)

[2] Guo LL, Dong GX, Zhang SH, et al. The effect of All-trans retinoic acid on the expression fo C-myc and the proliferation of vascular smooth muscle cells in atherosclerotic lesion of the rabbit carotid artery [J]. Fudan University Journal of Medical Sciences, 2006, 33 (4): 526-529(In Chinese)

[3] Zhang YB, Dong GX, Zhang SH. The effect for All-trans retinoic acid on the expression of NF-KB, P selection factor and VCAM-1 in atherosclerotic lesion of the rabbit carotid artery. Fudan University Journal of Medical Sciences, 2010, 37(5): 565-569(In Chinese)

[4] Ross R. Atherosclerosis-an inflammatory disease [J].N Engl J Med, 1999, 340(2):115-126

[5] Gerard C, Roll ins BJ. Chemokines and disease [J]. Nat Immunol, 2001,2(2) :108-115

[6] Rollins BJ. Chemokines[J].Blood, 1997, 90 (3) :909-928

[7] Geng XY, Chen YW. The research development on the role that Toll-like receptor plays in atherosclerosis [J]. Chinese Journal of Arteriosclerosis, 2008,16 (1) :78-80(In Chinese)

[8] Zhang XM, Dong GX, Zhang SH, et al. The effect of All-trans retinoic acid on the exprssion of P16 and PCNA after the balloon injury of rat thoracic aorta[J]. Chinese Journal of Arteriosclerosis, 2004, 12(2): 173-176(In Chinese)

[9] Hou L, Xu F, Han XN, et al. The effect of All-trans retinoic acid on the expression level of CyclinA and proliferation of aorta smooth muscle cells of the raised rat[J]. Foreign Medical Sciences Cardiovascular Diseases, 2004, 6: 356-359(In Chinese)

[10] Zhang WG, Don GX, Hou L, et al. The effect of All-trans retinoic acid on the proliferation and the expression of P21 of aorta smooth muscle cells of the raised rat[J].Journal of Xinxiang Medical College, 2003, 20(3) :163-165(In Chinese)

[11] Zhang WG, Dong GX, Han XN, et al. The effect of All-trans retinoic acid on the proliferation and the expression of CyclinE of aorta smooth muscle cells of the raised rat [J].Journal of Xinxiang Medical College, 2003, 20(5):322-323(In Chinese)

[12] Wen JK, Shi Y. The research on the regulation mechanism of the proliferation of vascular smooth muscle cells[J].Progress in physiological sciences, 1996, 27(2):149-152(In Chinese)

[13] Zou Y, Hu Y, Metzler B, et al. Signal transduction in atherosclerosis : mechanical stress2activated MAP kinases in vascular smooth muscle cells(review)[J]. Int J MoI Med, 1998, l(5) :827-834(In Chinese)

[14] Force T, Bonventre Jv. Growth factors and mitogen-activated protein kinases[J]. Hypertension, 1998, 31(Pt 2) :152-16l

[15] Maiti S, Chen X, Chen G. All-trans retinoic acid induction of sulfotransferases[J]. Basic Clin Pharmacol Toxicol, 2005,96(1):44-53

[16] Chen J, He B, Zheng D ,et al. All-trans retinoic acid reduces intima thickening after balloon angioplasty in atherosclerotic rabbits [J]. Chin Med J (Engl), 1999, 112(2):121-123

[17] An H, Zhang J. The research on ATRA treating the rat nephrosis caused by adriamycin [J].Journal of Sichuan University, 2007, 24(7): 56-67(In Chinese)

全反式维甲酸对兔动脉粥样硬化病灶中 MCP-1 和 TLR-4 表达的影响

任 娜 董果雄△ 张社华 张 滨 胡真真

(青岛大学医学院附属医院心 心内科 山东 青岛 266003)

摘要 目的:观察全反式维甲酸(ATRA)对兔颈动脉粥样硬化性病灶中内膜增生、MCP-1 及 TLR-4 表达的影响,探讨其可能的抗炎机制。方法:新西兰雄性大白兔随机分为 9 组(n=6):对照组(A、B、C)、治疗组(A、B、C)、假手术组(A、B、C)。除假手术组外,其余两组给予高脂饮食 2 周后,对照组及治疗组给予颈动脉内膜空气干燥术损伤颈动脉内膜,假手术组分离暴露颈动脉但不损伤内膜,治疗组术前 3 天给予 ATRA 灌胃,直至处死。术后分别于 7d、14d、28d 处死。采取颈动脉标本,对血管粥样硬化病变进行形态学观察及测定,采用免疫组化法检测 MCP-1 及 TLR-4 表达水平。结果:从形态学观察及免疫组化检测看,对照组较假手术组内膜明显增生,MCP-1 及 TLR-4 表达增多,治疗组内膜较对照组增生减轻,两种因子表达减少。结论:全反式维甲酸(ATRA)对兔颈动脉粥样硬化性病灶中的抗炎作用可能是通过抑制 MCP-1 及 TLR-4 等炎症因子的表达来发挥作用的。

关键词:维甲酸;炎症;MCP-1;TLR-4

中图分类号:R541.4 **文献标识码**:A **文章编号**:1673-6273(2012)05-823-06

作者简介:任娜,(1982-),女,硕士,主要从事动脉粥样硬化方面的研究

△通讯作者:董果雄,E-mail: qydgx@public.qd.sd.cn

(收稿日期:2011-07-16 接受日期:2011-08-10)