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All-Trans-Retinoic Acid Attenuates Neointima Formation with Acceleration of Reendothelialization in Balloon-Injured Rat Aorta

Retinoic acids may inhibit vascular smooth muscle cell proliferation, but may promote endothelial cell proliferation in cell culture. However, little data are available about the effects of all-trans-retinoic acid (ATRA) on endothelial regeneration and functional recovery in an experimental model of vascular injury. Accordingly, we investigated whether ATRA may attenuate neointima formation and accelerate endothelial regeneration with functional recovery in balloon-injured rat aorta. Twelve-week-old male Sprague-Dawley rats underwent endothelial denudation of the thoracic aorta by balloon injury. Fourteen rats were fed a standard rat pellet diet. Another 14 rats were fed ATRA (1.5 mg/day) for 2 weeks. The animals were killed on day 14 for organ chamber study and morphometric analysis. Rats in the ATRA group had a significantly improved acetylcholine-induced relaxation response than those in control group. However, endothelial independent response was not significantly different between the two groups. The extent of reendothelialization was markedly superior in the ATRA group compared with control group ($p<0.05$). Furthermore, neointima area and the ratio of neointima to medial area were significantly less in ATRA group than in control group ($p<0.05$). In conclusion, ATRA may accelerate endothelial regeneration with functional recovery, and attenuate neointima formation in balloon-injured rat aorta.

Key Words: *Tretinoin; Endothelium; Intima, Arterial*

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INTRODUCTION

Intimal hyperplasia plays a critical role in the progression of atherosclerotic lesions and restenosis after angioplasty (1-3). Blood vessel injuries induce proliferation and migration of smooth muscle cells and accumulation of extracelluar matrix, leading to neointima formation (4, 5). Endothelial cells may regulate the growth of the underlying smooth muscle cells and inhibit the formation of a hyperplastic neointima (4-8). Until now, many approaches to reduce postangioplasty restenosis have focused on retarding smooth muscle cell growth and other strategies have been used as well, such as vascular endothelial growth factor (7) and fibroblast growth factor (8). However, the pharmacologic studies of the past decade have been almost uniformly disappointing (9).

Currently, much interest is focused on the potential therapeutic benefits of retinoic acids (10-19). Retinoic acids play an important role in the control of growth and differentiation in the cardiovascular system (10). Retinoic acids may inhibit vascular smooth muscle cell proliferation (11-14), but may promote endothelial cell proliferation (18, 19). In addition, recent studies suggest that all-trans-retinoic acid (ATRA) may reduce neointimal mass and elicit positive remodeling of the injured rat carotid artery (12). However, little data are available about the effects of ATRA on endothelial regeneration and functional recovery in an experimental model of vascular injury. The aim of this study was therefore to determine whether ATRA accelerate endothelial regeneration with functional recovery and inhibit neointima formation in balloon-injured rat aorta.

MATERIALS AND METHODS

Animals

Twelve-week-old male Sprague-Dawley rats (350-450 g) were used for this study according to the protocol approved by the Institutional Animal Care and Use Committee. All rats were anesthesized with an intraperitoneal

injection of pentobarbital sodium solution (60 mg/kg). A 2F Fogarty catheter (Barxer Healthcare Corp, U.S.A.) was introduced through an arteriotomy in the external carotid artery and advanced to descending thoracic aorta. To produce deendothelialization injury of descending thoracic aorta, we inflated the balloon with 0.5 cc air and withdraw it three times from just diaphragm level to aortic arch under fluroscopic guide. After deendothelialization procedure, the rats were randomized to receive a standard rat pellet diet (n=14) or retinoic acid diet (n=14). Retinoic acid diet was administered as described previously (ATRA 1.5 mg/day, Sigma Chemical Co.) (20). All rats were killed two weeks after balloon injury, and used for organ chamber study or planimetric analysis of reendothelialization.

In vitro vasoreactivity

The denuded arterial segments were used for organ chamber studies. All drugs were obtained from Sigma Chemical Co (Sigma Chemical Co, St Louis, MO, U.S.A.). The arteries were rinsed in modified Krebs-Ringer bicarbonated solution (mmol/L): NaCl 118, KCl 4.7, $NaHCO_3$ 25.0, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, Ca-EDTA 0.016 and D-glucose 11.1 (pH 7.4). Loose connective tissue in the adventitia was removed and the vessel was cut into rings (3 to 4 mm in length). The rings were suspended in an organ bath containing Krebs' buffer aerated with 95% O_2-5% CO_2. The rings were connected to a force transducer to measure isometric tension. Resting tension was increased stepwise to reach the final tension of 1.0 g and the rings were allowed to equilibrate for at least 30 min. Phenylephrine (10^{-9} to 10^{-5} mol/L) was applied in a cumulative manner, and the concentration of phenylephrine that caused a half-maximal response was used to precontract the rings. In the presence of precontraction, vasodilator response was determined by cumulative adding acetylcholine (10^{-9}-10^{-5} M) or nitroprusside (10^{-9}-10^{-5} M). Relaxation to acetylcholine was also obtained with preincubation of NG-monomethyl-L-arginine (10^{-4} M).

Evaluation of intimal hyperplasia

After completion of the in vitro vasoreactivity, each ring was placed in 10% buffered formalin solution. Vessels were embedded in paraffin and serially sectioned (5 μm) for morphometric analysis. Neointimal thickening was assessed in terms of the intima-to-media area ratio after staining with hematoxylin and eosin or elastic-trichrome-stained arterial sections by one analyst. Morphometric analysis of each arterial segment was performed with a computerized sketching program (Image-Pro Plus Version 3.0, Media Cybernetics, Denmark) by a single examiner who was blinded to the treatment regimen. At least three sections of each vessel were examined, and measurements were averaged for statistical analysis. The cross-sectional areas of the media and neointima were measured.

Macroscopic evaluation of reendothelialization

Evans blue dye (0.5%, 0.5 cc) was injected via tail vein 30 min before they were killed (n=14). Perfusion fixation was performed using 100% methanol after perfusion of phosphate-buffered saline and the denuded segments were dissected longitudinally. Planimetric analysis of the photograph of the harvested arterial segment stained with Evans blue dye was performed with a computerized sketching program (Image-Pro Plus Version 3.0, Media Cybernetics, Denmark) by one analyst who was blinded to the treatment regimen. The initially denuded area was defined as the total surface area of the harvested arterial segment. The reendothelialized area was defined macroscopically as the area that was not stained with Evans blue dye.

Statistical analysis

Statistical analysis was performed using SPSS 7.5 for Windows. Results were expressed as mean ± SEM. Differences between groups were evaluated using two-tailed, unpaired Students t test. A p value of <0.05 was considered significant.

RESULTS

Vasodilator responses

Acetylcholine induced relaxation was more prominent in vessels from ATRA group than that in segments in control group, achieving a statistical significance at concentrations of 10^{-9} M and 10^{-5} M (Fig. 1). However, there was no difference between the two groups in vasodilator response to sodium nitroprusside (Fig. 2). In the presence of N^G-monomethyl-L-arginine, acetylcholine-induced relaxation response was similar between the two groups (Fig. 1).

Neointimal thickening

Morphometric analysis showed that the neointimal area and the ratio of neointimal area to medial area were significantly less in ATRA group than in control group (Table 1). For the two-week control group, intima-to-

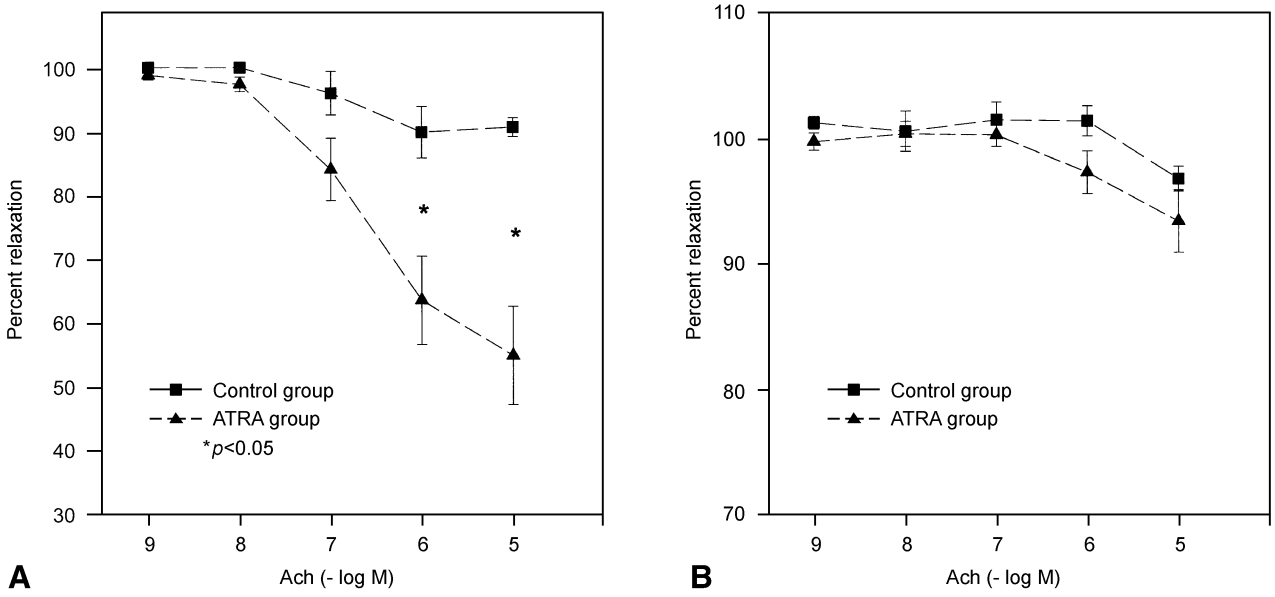

Fig. 1. Cumulative dose-response curves to acetylcholine during precontraction to phenylephrine. A: Acetylcholine-induced relaxation is more prominent in ATRA group than in control group. B: Pretreatment with NG-nitro-L-arginine attenuates acetycholine-induced relaxation with no difference between the two groups.

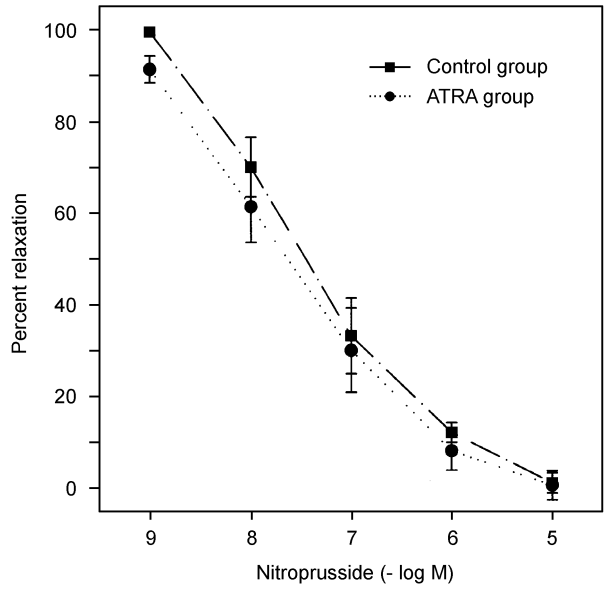

Fig. 2. Cumulative dose-response curves to nitroprusside during precontraction to phenylephrine. Nitroprusside-induced relaxation response is similar between the two groups.

media area ratio was 0.199±0.022 whereas, intima-to-media area ratio for ATRA group was 0.104±0.008. There was no significant difference in the medial area between the two groups (Fig. 3).

Extent of reendothelialization

Planimetric analysis revealed no significant difference between the total area of initial balloon injury in ATRA group and that of control group (Table 2). Typical examples of the macroscopic appearance of Evans blue dye-stained segment from control are shown in Fig. 4, which shows the characteristic pattern of being localized only to one side of the vessel (21) and represents an increased renendothelialization in ATRA group compared with that in control group. The reendothelialized area in the two-week control group was 175.0±14.2 mm^2; in contrast, the reendothelialized area in the two-week ATRA group was 245.0±24.8 mm^2 (p<0.05) (Table 2).

Table 1. Effects of all-trans-retinoic acid on myointimal proliferation after balloon injury

	ATRA group (n=7)	Control group (n=7)
Body weight, g	400±5	398±6
Thoracic aorta		
Intima area, mm^2	0.065±0.006	0.122±0.014*
Area of media, mm^2	0.615±0.019	0.614±0.017
Intima/media ratio, %	10.4±0.8	19.9±2.2*

ATRA, all-trans-retinoic acid; *p<0.05

Table 2. Effects of all-trans-retinoic acid on endothelial regeneration after balloon injury

	ATRA group (n=7)	Control group (n=7)
Total area, mm^2	612.0±37.8	629.0±46.8
Regenerated area, mm^2	245.0±24.8	175.0±14.2*
Regenerated area/total area, %	40.0±2.4	28.0±2.4*

ATRA, all-trans-retinoic acid; *p<0.05

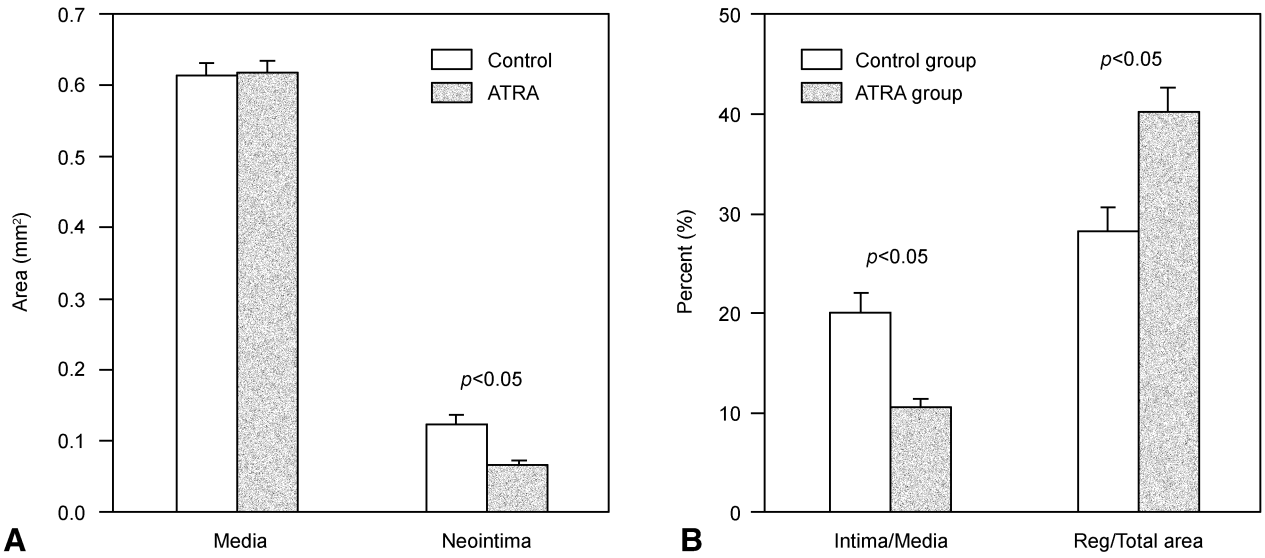

Fig. 3. Effects of all-trans-retinoic acid on neointima formation (**A, B**) and endothelial regeneration (**B**). Neointima area is smaller in ATRA group than in control group ($p<0.05$). However, reendothelialization is accelerated in ATRA group than in control group ($p<0.05$). Reg denotes reendothelialized area.

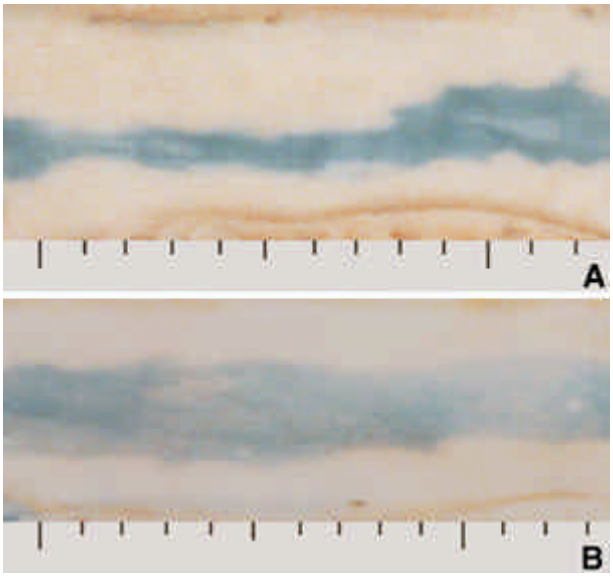

Fig. 4. Evans blue stained area represents nonendothelialized area, whereas reendothelialized area appears white. Extent of reendothelialization is greater in ATRA-treated segment (**A**) than in control segment (**B**).

DISCUSSION

The major findings of this study are that 1) ATRA may restore abnormal response of previously denuded rat aorta to endothelium-dependent agonist, 2) it may promote reendothelialization and 3) it may attenuate neointimal thickening in balloon-injured rat aorta. These results suggest that ATRA may be useful to reestablish normal endothelial function and inhibit neointima formation after balloon angioplasty.

Few studies have evaluated the effects of retinoic acid derivatives on endothelial function and angiogenic activity (16-19, 22). Unfortunately, controversy still exists, and both stimulatory (17, 18) and inhibitory (16) activities of these agents on angiogenic activity (endothelial cell proliferation and migration) have been reported. In this study, ATRA accelerated endothelial regeneration and functional recovery in a balloon injury model of rat aorta. In previous studies, retinoids were shown to be inhibitors of angiogenesis in cell culture system (16). However, a recent study showed that retinoic acids have strong positive effects on angiogenesis in the presence of inflammation (17). The different effects of these agents on endothelial proliferation and migration may reflect the importance of microenvironment.

Smooth muscle migration and proliferation plays a key role in the development of restenosis after angioplasty (1-3). A number of studies have been focused on inhibiting smooth muscle cell proliferation but only a few studies were successful in clinical trials (9). Recently, several studies suggested that retinoic acids can affect the proliferation, migration and differentiation of smooth muscle cell in vitro and reduce neointima formation induced by vascular injury (12). Retinoic acids may suppress mitogenesis in cultured cells, but in some circumstances, it can stimulate rather than suppress mitogenesis, making it difficult to assess its growth-regulatory properties (10-19). These divergent properties to stimulate or suppress may depend on the underlying pathophysiologic conditions. Further studies may be needed to ascertain these issues.

The present results showed that ATRA accelerated the

regrowth of endothelium and restored endothelial function in injured aorta, supporting retinoic acids as a key factor in the regulation of cell growth and differentiation (10). However, the mechanisms underlying the acceleration of reendothelialization are not known. We speculate that through interaction between ATRA and some growth factors, such as vascular endothelial cell growth factor and basic fibroblast growth factor, endothelial cell regrowth may be accelerated.

The role of retinoids in the regulation of mitogenesis may be rather complex. They have the ability to either stimulate or suppress mitogenesis, depending on the experimental conditions. The exact mechanisms of ATRA that reduce neointima formation remain uncertain, but several explanations are possible. ATRA has been shown to suppress smooth muscle cell migration in vitro through inhibition of the AP-1 dependent pathway (22). In addition, ATRA seems to regulate several apoptotic genes, including *bcl*-2, leading to apoptosis of vascular smooth muscle cells (23).

The present results suggest that ATRA may restore endothelial function of the rat thoracic aorta after balloon injury. Furthermore, recent studies indicate that ATRA may reduce neointimal mass and elicit favorable geometric remodeling of the injured rat carotid artery. ATRA, which is a relaively safe agent, is widely used in various clinical conditions (24). ATRA may certainly be an attractive candidate for clinical trials in preventing restenosis, however, a number of agents tested in animal models were not successful in clinical trials. Therefore, further studies are necessary to determine the efficacy of this agent in preventing restenosis.

Several potential limitations need to be addressed. First, the dose of ATRA used in this study is rather high, requiring to define a dose-response of ATRA that reduces neointima formation. Second, the rat aorta injury model is not adequate for the evaluation of restenosis mechanisms, and therefore the effects of ATRA observed in this study may not be generalized to other animal models or human.

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