**All-trans-Retinoic Acid Limits Restenosis After Balloon Angioplasty in the Focally Atherosclerotic Rabbit**

**A Favorable Effect on Vessel Remodeling**


**Abstract**—All-trans-retinoic acid (atRA) has potent in vitro effects on a number of processes involved in vascular injury and repair, such as modulating smooth muscle cell (SMC) proliferation and inducing SMC differentiation, and may play an important role in the in vivo response to vascular injury. We hypothesized that atRA would limit restenosis after balloon angioplasty through SMC-modulated changes in plaque size and vessel geometry. Balloon angioplasty was performed on rabbits with focal femoral atherosclerosis randomized to treatment with atRA or saline. At 28 days after balloon angioplasty, minimum luminal diameter was significantly larger in the atRA group (1.24 ± 0.17 versus 1.12 ± 0.22 mm, \( P = 0.02 \)). Histomorphometry confirmed a larger lumen area (0.51 ± 0.20 versus 0.34 ± 0.13 mm\(^2\), \( P = 0.004 \)) in the atRA group, with no difference in absolute plaque area. Internal elastic lamina and external elastic lamina areas were significantly larger in the atRA group (0.89 ± 0.27 versus 0.66 ± 0.24 mm\(^2\), \( P = 0.001 \), and 1.29 ± 0.38 versus 0.98 ± 0.32 mm\(^2\), \( P = 0.001 \), respectively). Vessel sections exhibited significantly more \( \alpha \)-actin and desmin immunostaining (\( P = 0.01 \)) in the atRA-treated group. No differences in early cellular proliferation and collagen content were detected with the use of bromodeoxyuridine. In this atherosclerotic model of vascular injury, atRA limits restenosis after balloon angioplasty by effects secondary to overall vessel segment enlargement at the angioplasty site rather than by effects on plaque size or cellular proliferation. Increased \( \alpha \)-actin and desmin immunostaining suggest a possible role for phenotypic modulation of SMCs in this favorable remodeling effect. *(Arterioscler Thromb Vasc Biol. 2000;20:89-95.)*

**Key Words:** retinoic acid ■ restenosis ■ remodeling ■ angioplasty ■ smooth muscle cells

Despite extensive investigation, the effectiveness of percutaneous transluminal coronary angioplasty remains limited by restenosis at the angioplasty site.\(^1\)\(^-\)\(^3\) Restenosis is thought to involve a complex interaction of biological processes initiated by arterial injury with resultant platelet deposition; thrombin generation; release of chemotactic, vasoactive, and mitogenic factors; migration and proliferation of smooth muscle cells (SMCs); and extracellular matrix synthesis. These processes combine to produce neointimal plaque growth and remodeling of vascular architecture resulting in variable degrees of arterial narrowing.\(^4\)\(^-\)\(^6\)

All-trans-retinoic acid (atRA), a naturally occurring metabolite of vitamin A, has potent in vitro effects on a number of processes thought to be involved in the vascular response to injury. For example, atRA has been shown to induce differentiation of multipotential embryonal carcinoma cells to express multiple SMC characteristics, including changes in cell morphology, responses to contractile agonists, and expression of SMC-specific isoforms of \( \alpha \)-actin and myosin heavy chain.\(^7\) Furthermore, atRA induces cultured SMCs to assume a more differentiated contractile phenotype, as assessed by \( \alpha \)-actin expression.\(^8\) By inducing SMCs to differentiate into a mature “contractile” phenotype, atRA could potentially limit neointimal formation and possibly hinder pathological vascular remodeling after vascular injury by inhibiting the secretion of growth factors, chemokines, and the elaboration of extracellular matrix by “dedifferentiated” (\( \alpha \)-actin-negative) SMCs. Other studies suggest that retinoids not only affect the phenotypic modulation of SMCs but also inhibit cellular proliferation,\(^9\) increase elastin synthesis,\(^10\) inhibit collagen synthesis,\(^11\) and stimulate metalloproteinase inhibitor production by fibroblasts,\(^12\) all of which may have important effects on matrix dynamics and stability of the atherosclerotic plaque. Retinoids have a number of other actions, including immunomodulatory actions,\(^13\) modification of cytokine-induced responses\(^14\) affecting nitric oxide production in macrophages and cultured SMCs,\(^15\)\(^16\) and reduction of tissue factor/factor VIIa–dependent arterial thrombus...
formation. Finally, functional retinoid receptors have been identified in cultured SMCs, and atRA has been shown to inhibit SMC growth in vitro.\textsuperscript{17,18} In vitro, atRA differentiates leukemic promyelocytes into mature cells, and complete remission rates of 90% have been observed in phase 2 studies of patients with acute promyelocytic leukemia by use of atRA at doses of 1.0 to 1.5 mg/kg.\textsuperscript{20–22} To date, few in vivo studies have addressed the role of atRA in the response to arterial injury.\textsuperscript{23} On the basis of its effects on modulating SMC proliferation and differentiation, we hypothesized that atRA would limit restenosis after balloon angioplasty (BA) in the focally atherosclerotic rabbit either by changes in plaque size or by vessel geometry.

**Methods**

**Induction of Focal Femoral Atherosclerosis**

Thirty-six male New Zealand White rabbits weighing 4.0 ± 0.4 kg were anesthetized by intramuscular injection of ketamine (35 mg/kg) and xylazine (5 mg/kg). Bilateral focal atherosclerosis was induced in 1- to 2-cm femoral artery segments by air desiccation endothelial injury, followed by a 2% cholesterol/6% peanut oil diet for 28 days as described previously.\textsuperscript{24–26} The rabbits were housed according to Animal Welfare Act specifications, and all surgical procedures were performed using a sterile technique and general anesthesia.

**Drug Administration**

Twenty-six animals were randomly assigned to treatment with atRA or placebo for 28 days. The atRA was administered at a dose of 25 mg in 1.5 mL of vegetable oil (\(~6\text{ mg/kg per day}\) by oral intubation daily for the 3 days before and for 28 days after BA (n = 11). Control animals (n = 15) received vehicle oil alone by the same method. Blood samples for serum atRA levels were obtained when the animals were euthanized. Blood was stored in vacuum tubes shielded from light and centrifuged within 2 hours. The serum fraction was isolated and stored at \(-70^\circ\text{C}\). Samples were shipped on dry ice to the M.D. Anderson Cancer Center, University of Texas, Houston, for analysis by high-pressure liquid chromatography (kindly provided by Dr Herbert A. Fritsche). This dose of atRA has been shown to be of moderate teratogenicity in rabbits\textsuperscript{26} and was slightly greater (3 to 4 times) than the dose used in human acute promyelocytic leukemia.\textsuperscript{21} This regimen of atRA has been shown to produce peak and trough atRA concentrations in rabbit serum of 100 and 5 ng/mL, respectively.\textsuperscript{26} All animals received heparin (150 U/kg) by intravenous bolus immediately before BA.

**Balloon Angioplasty**

After induction of anesthesia, the right carotid artery was exposed through a midline neck incision. A 5F Berman catheter (Arrow International, Inc) was inserted via an arteriotomy and advanced into the descending aorta under fluoroscopic guidance. Baseline angiography was performed by using a Siemens Optilux angiographic system, and images were recorded on 35-mm cineradiographic film with the use of 5 mL Hexabrix (39.3% ioxaglate meglumine/19.6% ioxaglate sodium injection) diluted with 5 mL of sterile saline. A grid with 5-mm markings was used as an internal calibration standard. BA was performed by use of a 0.014-in., guidewire and a 2.5-mm balloon dilation catheter with three 30-second 10-atm inflations. The same protocol was repeated for the contralateral femoral artery. The catheter and vascular sheath were then removed, the carotid artery was ligated, and the wound was closed. Rabbids (n = 26) were maintained for 28 days after BA and fed normal rabbit chow. Final angiography was performed with the left carotid artery and the technique described above just before euthanasia.

**Angiography**

Angiograms were analyzed on a Sony coronary angiography diagramming and reporting system. Minimum luminal diameter (MLD) of the femoral artery segment was measured before BA, after BA, and at 28 days in a blinded fashion by 2 observers. Intraobserver and interobserver variability were 0.95 and 0.94, respectively. All images were obtained in 1 angiographic plane and calibrated with a grid.

**Pressure Perfusion and Specimen Preparation**

At 28 days after BA, final angiography was performed, and the animals were euthanized by an overdose of sodium pentobarbital. The distal aorta and iliofemoral segments were perfused at physiological pressure (100 mm Hg and 22°C) with 100 mL of 4% buffered paraformaldehyde. Segments of the femoral artery (\(~4\text{ to } 5\text{ cm}\) were harvested bilaterally, and the proximal and distal ends were marked with silk ligatures. The specimens were postfixied in 4% buffered paraformaldehyde and prepared for light microscopy.

**Histopathology and Immunostaining**

Each femoral artery segment was cut in cross section at 3- to 4-mm intervals, dehydrated in 70% ethanol and xylene, and embedded in paraffin. Serial 5-μm sections from each 3- to 4-mm segment were stained by use of the Movat technique. The section with the greatest luminal narrowing was identified for each angioplasty site, and quantitative histomorphometry was performed in a blinded fashion with an Olympus AH-2, Vanox-S microscope in association with Mocha image analysis software and a 486/80-based PC. The luminal border, internal elastic lamina (IEL), and external elastic lamina (EEL) were traced at the same magnification, and the image was calibrated to a 1-mm grid. Overall vessel size was measured as the total area bounded by the EEL. Plaque size was measured as the area bounded by the IEL minus the lumen area.

**Injury Score**

Each femoral artery segment used for quantitative histological evaluation was also evaluated for the extent of injury by using a semiquantitative scale. The scoring system was as follows: 0 indicates sections with IEL intact and media compressed but not lacerated; 1, EL lacerated, with media compressed but not lacerated; 2, IEL and media lacerated, with EEL intact; and 3, large transmural laceration involving the EEL.

**SMC Characterization**

The characterization of the “quiescent” contractile SMC phenotype was assessed by α-actin and desmin immunostaining with the avidin-biotin-peroxidase method (Vector Laboratories). Sections from all analyzed vessels were stained with anti-SM-specific α-actin antibody (clone HHF-35, undiluted, Enzo Inc), and quantitative analysis was performed in a blinded fashion by using the microscopic image analysis system described above. Digitized images were analyzed for areas of positive staining with Image-Pro software, version 3.0 (Media Cybernetics). A color threshold mask for immunostaining was defined to detect the brown color by sampling, and the same threshold was applied to all the vessels. For each arterial section, the area (mm\(^2\)) of the intima and media with α-actin staining was determined. One adjacent section from each vessel was also stained with anti-desmin antibody (clone 33, 1:80 dilution, BioGenex Laboratories), and semiquantitative analysis was performed by a blinded observer using a 0 to 4 scaling system (0, indicating absence of staining, to 4, indicating diffuse heavy staining). Negative controls for desmin and α-actin stains were performed on vessel sections with omission of the primary antibody. Serial vessel segments were analyzed for collagen content by use of Sirius red staining (F3BA Sirius red, Cell Point), and polarized microscopy and a semiquantitative scale were used as described above.

**Bromodeoxyuridine Labeling for Cellular Proliferation**

Ten rabbits (n = 5 for atRA, n = 5 for controls) received 50 mg bromodeoxyuridine (BrdU, Sigma Chemical Co) by subcutaneous injection twice daily for 3 days after balloon dilatation to investigate the effect of atRA on early cellular proliferation after BA. Vessels were prepared as described above. Sections were stained for BrdU-positive nuclei by use of a mouse monoclonal anti-BrdU antibody (Dako, Inc) and an anti-mouse IgG “ABC” kit (Vector Laboratories). BrdU-positive immunostaining in the intima and media was analyzed by the image analysis system described above. Analysis of

**References**

1. Dr. Herbert A. Fritsche. This dose of atRA has been shown to be of moderate teratogenicity in rabbits. 2. IEL and media lacerated, with EEL intact; and 3, large transmural laceration involving the EEL.
each section was performed twice to assure reproducibility. Cumulative BrdU incorporation is reported as total number of BrdU-positive nuclei per millimeter.

**Statistical Analysis**

Data are reported as the number of femoral arteries in each experimental group and expressed as the mean±SD. Angiographic and histological differences between the 2 treatment groups were analyzed by the Student t test. For nonparametric data, such as the injury score and desmin score, differences between the 2 treatment groups were detected by use of a 2-tailed Mann-Whitney U test. A value of $P≤0.05$ was considered significant. Correlation of IEL and EEL to plaque area of the atRA and control groups was performed by regression analysis.

**Results**

**Drug Levels**

At euthanasia, atRA concentrations in the blood were 44±30 ng/mL in the atRA-treated animals compared with 0 ng/mL in the control animals ($P=0.004$).

**Angiography**

The angiographic data are summarized in Figure 1. MLD was similar in the 2 treatment groups at baseline (pre-BA) and immediately after BA (post-BA). Vessels with abrupt closure at the angioplasty site immediately after BA were excluded from analysis (n=1 for atRA, n=4 for controls), resulting in 21 vessels (from 11 rabbits) analyzed in the atRA group and 26 (from 15 rabbits) in the control group. The MLD 28 days after BA was significantly larger in the atRA group than in the control group (0.51±0.20 versus 0.34±0.13 mm², $P=0.004$). Absolute plaque area was similar in the atRA and control groups (0.38±0.19 versus 0.32±0.14 mm², $P=0.15$). The IEL and EEL areas were significantly larger at the angioplasty site in the atRA group than in the control group (0.89±0.27 versus 0.66±0.24 mm², $P=0.001$, and 1.29±0.38 versus 0.98±0.32 mm², $P=0.001$, respectively). Figure 3 demonstrates the correlation of IEL area (panel A) and EEL area (panel B) to plaque area (in mm²) in the atRA and control groups (atRA group, IEL = 0.96±plaque area + 0.53, $P=0.001$; control group, IEL = 0.50±plaque area + 0.18, $P=0.0001$; atRA group, EEL = 0.53±plaque area + 0.71, $P=0.0001$; and control group, EEL = 0.9±plaque area + 0.37, $P=0.0001$). At similar plaque areas, both IEL and EEL areas were greater in the atRA group, demonstrating an effect on favorable remodeling to a larger vessel size, which translates into a larger lumen size as measured by both angiography and histomorphometry. Vessel injury scores were not statistically different between atRA-treated and control animals (1.3±1.2 versus 0.83±0.92, respectively).

**Cellular Proliferation**

Cellular proliferation, as measured by cumulative BrdU incorporation at 72 hours post-BA, was similar in the atRA (n=10 vessels) and control (n=10 vessels) groups. The mean number of BrdU-positive cells was 799±371 cells/mm² in the atRA group versus 988±465 cells/mm² in the control group ($P=0.39$).

**α-Actin, Desmin, and Collagen Staining**

Results of the α-actin and desmin staining are depicted in Figure 4, and representative photomicrographs are shown in Figure 5. Vessel sections in the atRA-treated group exhibited significantly more α-actin staining than did the control group (0.23±0.11 versus 0.15±0.06 mm², $P=0.01$). Similarly, desmin immunostaining by qualitative scoring was higher in the atRA group than the control group (mean desmin score, 1.90±0.76 versus 1.35±0.55, $P=0.01$). Collagen staining was similar in the 2 groups (qualitative score, 0.39±0.13 versus 0.39±0.14 mm²).

**Discussion**

The results of the present study performed in the double-injured focally atherosclerotic rabbit demonstrate that both angiographic and histological lumen areas were larger at 28
days after BA in atRA-treated animals compared with control animals. This effect on lumen size was secondary to a larger overall vessel size at the angioplasty site rather than the result of a smaller plaque area or an inhibition of early cellular proliferation.

Although strategies to limit restenosis have focused on inhibition of SMC proliferation and/or thrombosis, more recent observations suggest that changes in overall vessel size at the injury site may contribute to restenosis. “Unfavorable” or negative vascular remodeling, defined as a decrease in EEL area, has been reported by serial intravascular ultrasound studies to contribute to the late luminal narrowing associated with restenosis in humans. The determinants of vascular remodeling to an overall smaller vessel size after arterial injury remain poorly understood. Coronary stenting provides a mechanical strategy to potentially reduce this problem. Recently, prolonged pretreatment with the antioxidant probucol was shown to reduce restenosis in humans, primarily through improved vascular remodeling after angioplasty. The authors speculated that the antioxidant effects of probucol may have modified vascular remodeling through decreased SMC activation, migration, and proliferation, resulting in limited matrix degradation and new collagen deposition. Our data suggest that atRA may also favorably affect vascular remodeling after vessel injury. The cellular and molecular mechanisms involved in this effect on vascular dimensions are unknown. Our observation that atRA did not alter cumulative BrdU incorporation at 3 days after BA provides evidence that the in vivo effect on lumen size is not predominantly due to inhibition of early cellular proliferation. Previous studies in our laboratory have suggested that the peak SMC proliferation occurs 3 days after BA, with return of cellular proliferation to baseline at 7 days. The finding of similar rates of proliferation in the treatment and control groups is contrary to observations in cell culture, where atRA has been shown to inhibit proliferation of SMCs. Additional studies are necessary to determine whether this apparent contradiction may be due to species differences or different behavior of SMCs in culture versus SMCs in vivo after vascular injury. In addition, other mediators of SMC proliferation in vivo may not be affected by atRA. It is also possible that inhibition of SMC mitogenesis is only seen at higher doses than we used in vivo. Our dose of 6 mg/kg per day is 3 to 4 times the dose used in human clinical trials in acute promyelocytic leukemia. In vitro, atRA inhibits

Figure 3. A, Regression analysis of the correlation of IEL area to plaque area in the atRA-treated and control groups. Note that despite similar mean plaque areas in the groups, IEL area is larger in the atRA group. B, Regression analysis of the correlation of EEL area to plaque area in the atRA-treated and control groups. Note that despite similar mean plaque areas in the groups, EEL area is larger in the atRA group.

Figure 4. A, Bar graph showing desmin immunostaining using qualitative analysis in vessel sections at the angioplasty site in the atRA-treated and control rabbits 28 days after BA. Values are expressed as mean scores (0, indicating no staining, to 4, indicating heavy diffuse staining). B, Bar graph showing α-actin immunostaining in vessel sections at the angioplasty site in the atRA-treated and control rabbits 28 days after BA. Desmin scored using semiquantitative visual methods; actin, digitized computer method.
platelet-derived growth factor–stimulated [3H]thymidine uptake in a dose-dependent manner, being maximal at $2 \times 10^{-6}$ mol/L.\textsuperscript{19}

Another possible mechanism by which atRA produces its favorable effect on remodeling might be by altering the elastic properties of the vessel wall and/or affecting vascular tone.\textsuperscript{9} In the present study, the luminal dimensions of the reference segments upstream from the angioplasty site were similar in both the atRA and control groups when examined by serial angiography, arguing against a generalized increase in vessel caliber through modulation of vascular tone, vessel elasticity, or blood pressure. Retinoids may also modulate collagen and elastin production at the angioplasty site in response to injury, resulting in a favorable effect on the healing response.\textsuperscript{9–11} However, our data did not demonstrate a difference in total vessel collagen content as measured by Sirius red staining, arguing against a significant effect on collagen metabolism.

A further interesting possibility is that atRA has important effects on the manifestation of SMC phenotype in vivo. The maintenance of SMCs in a “quiescent” phenotype might in part explain the favorable effect we observed on overall vessel size of the injured segment. There is extensive evidence showing that phenotypic modulation of SMCs is important in the response to arterial injury. Phenotypic modulation involves conversion of SMCs from a quiescent, differentiated, “contractile” phenotype to a less differentiated “secretory” phenotype characterized by an increase in growth responsiveness and enhanced secretion of extracellular matrix proteins.\textsuperscript{39} atRA has been shown in a variety of cell culture
experiments to impact a variety of these processes, ultimately promoting SMC phenotypic differentiation, resulting in increased collagen and metalloproteinase inhibitor production, which may then permit favorable enlargement of the injured segment.7–14 In support of this hypothesis, in the atRA-treated group, we observed significantly more α-actin and desmin staining: proteins were expressed to a greater degree by cells in the quiescent “contractile” phenotype. Our observations, however, do not establish a causal relation or chronicle the time course of SMC α-actin and desmin expression after injury. We believe that this would be an interesting focus for future studies of the potential beneficial effects of atRA on arterial repair.

The present study analyzed the effects of atRA on restenosis in this model by using both an in vivo method (angiography) and an in vitro method (histomorphometry). Despite perfusion with paraformaldehyde at physiological pressure, vessel shrinkage does occur during dehydration and processing before paraffin embedding. All vessels in the atRA and control groups are subject to this. A discrepancy between the angiographic MLD and the histomorphometry clearly exists. However, the directional changes in lumen size by both measures (1 in vivo and 1 in vitro) are concordant. Therefore, we assert that conclusions drawn on these data are valid. A further limitation of the present study was the fixed dose used in the experiment. The dose chosen was slightly higher than that used in human trials of atRA in the treatment of acute promyelocytic leukemia and was a dose shown to be of moderate teratogenicity in rabbits. A complete dose-response study of the drug is beyond the scope of the present investigation.

In conclusion, atRA resulted in a larger luminal diameter 28 days after BA, as determined by both angiography and histomorphometry in the hypercholesterolemic, focally atherosclerotic rabbit model of restenosis. The larger luminal diameter was secondary to an overall larger vessel size of the injured segment without a demonstrable effect on plaque size or early cellular proliferation. atRA resulted in no difference in early cellular proliferation or vessel collagen content but did result in higher α-actin and desmin staining, suggesting a role of phenotypic modulation in producing the favorable vessel enlargement after vascular injury.

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References


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