Acyclic Retinoid Inhibits Neointima Formation Through Retinoic Acid Receptor Beta-Induced Apoptosis

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Objectives—Acyclic retinoid (ACR) is a synthetic retinoid with a high safety profile that has been pursued with high expectations for therapeutic use in prevention (recurrence) and treatment of malignancies. With the objective of addressing the therapeutic potential in the cardiovasculature, namely neointima formation, effects of ACR on neointima formation and the involved mechanisms were investigated.

Methods and Results—ACR was administered to cuff-injured mice which showed inhibition of neointima formation. Investigation of involved mechanisms at the cellular and molecular levels showed that ACR induces apoptosis of neointimal cells and this to be mediated by selective induction of retinoic-acid receptor β (RARβ) which shows growth inhibitory and proapoptotic effects on smooth muscle cells.

Conclusion—We show that ACR inhibits neointima formation by inducing RARβ which in turn inhibits cell growth and induces apoptosis. The retinoid, ACR, may be potentially exploitable for treatment and prevention of neointima formation. (Arterioscler Thromb Vasc Biol. 2007;27:1535-1541.)

Key Words: smooth muscle cell ■ cell growth ■ retinoid

Retinoids are natural and synthetic derivatives of vitamin A which modulate growth, apoptosis, and differentiation of cells.1 Many retinoids have been pursued from therapeutic standpoints with the aim of chemotherapy of cancer, leukemia, and prevention of atherosclerosis.2 The biological effects of retinoids are mainly mediated by their receptors, retinoic-acid receptor (RAR) and retinoic-X receptor (RXR).3 RAR interacts with both all-trans retinoic acid (ATRA) and 9-cis retinoic acid (9cRA), and has 3 isoforms (α, β, and γ).2

Among the retinoid receptors, RARβ plays a critical role in mediating the effects of retinoid on cell growth and tumor inhibition in various cancer cells.3,4 RARβ expression diminishes with development of esophageal squamous cell carcinoma.4 Induction of RARβ by ATRA and overexpression of RARβ also play important roles in mediating the growth-inhibitory effects of ATRA in human lung squamous cell carcinoma.3 These findings indicate that modulation of expression of RARβ critically regulates cell growth inhibition. The role of RARβ in vascular SMCs (smooth muscle cells), however, has not been previously addressed.

Acyclic retinoid (ACR) is a novel synthetic RAR-selective retinoid (supplemental Table I) which has been shown to inhibit the growth of hepatoma cells in vivo and in vitro.5 ACR has been shown to inhibit the recurrence of hepatocellular carcinoma with oral administration in clinical trials, and has a high safety profile causing less side effects than other retinoids.6 Recently, ACR has been shown to preferentially act on and to elicit biological effects mainly through RARβ.7,8 At the cellular level, ACR induces apoptosis in hepatoma cells.9,10 ACR is therefore well-defined and safe, and is thus an attractive retinoid for therapeutic use. However, its effects on vascular remodeling have not been studied.

In the present study, we investigated whether ACR shows effects on neointima formation. We also addressed the mechanism of action of ACR in the vasculature, namely through effects on expression of RARs in SMCs with a focus on the biological role of RARβ. We show that expression of RARβ as induced by ACR exerts inhibitory effects on vascular remodeling through regulation of SMCs. We further show that induction of RARβ expression stimulates apoptosis. Collectively, we show that ACR may be a promising therapeutic agent against vascular disease, namely neointima formation.

Materials and Methods

Murine Cuff Injury Analysis

Nine-week-old male C57BL/6N mice (Oriental Yeast) underwent cuff-placement surgery in the right femoral artery as described.11,12 ACR and ATRA were emulsified in soybean oil. ACR, ATRA, and soybean oil (as vehicle control) were administered from 1 week before the procedure up to 5 weeks after the procedure. Agents were...
administered orally with a stomach tube (n=40; 10 from the vehicle control group, 10 from the ATRA group, 10 from the ACR low group, and 10 from the ACR high group). Animals were euthanized after the administration period and perfused first with phosphate-buffered saline (PBS) and then with neutral buffered formalin. Thereafter, the cuffed artery was removed. The segment of the artery was stained by Elastica van Gieson staining as described. We assessed areas of the neointima around the cuff by morphometric analysis as described previously. Experiments were reproduced in at least 3 other occasions.

Please see supplement, available online at http://atvb.ahajournals.org, for additional Materials and Methods.

**Results**

**ACR Inhibits Vascular Remodeling**

We first investigated the effects of ACR on vascular remodeling by the cuff-injury model. We administered ACR, ATRA (as control), or soybean oil (as vehicle control) to wild-type mice with cuffed femoral arteries. After the administration period, we assessed the neointima by histological methods.

In the groups treated with ACR, area of the neointima around the cuff was attenuated as compared with those in the groups treated with ATRA or vehicle control. ACR attenuated formation of the neointima in a dose-dependent manner, with those administered 200 mg/kg showing greater effect than those administered 100 mg/kg. Area of the neointima was composed mainly of SMCs and SMC-like cells as shown by SM actin staining (Figure 1A). To assess the effect on the neointima in a quantitative manner, the intima/media ratio, which is an analytic parameter of the neointima, was used. The intima/media ratio of the groups administered ACR was less than that of the ATRA-administered group or the vehicle control group; of note there was no difference in the medial area among groups (Figure 1B). ACR therefore attenuated vascular remodeling by reduction of neointimal SMCs in a dose-dependent manner.

It is noteworthy that although ATRA has been reported to attenuate neointima formation in the vascular injury model, the effects of synthetic retinoids compared with ATRA on vascular remodeling have not been studied in vivo. Moreover, retinoid activity was assessed by measuring serum alkaline phosphatase (ALP) which is a measure of effects on bone resorption. The relative levels of ALP when treated with 10 mg/kg of ATRA were at levels similar to that of ACR at 100 mg/kg, which were the dosages used for direct comparison in the present study (data not shown). Dosage of ACR at 100 mg/kg was therefore more potent than that of ATRA at 10 mg/kg.

Further, we examined adverse effects of retinoid administration. There was no significant difference in body weight
gain or bone fracture during the experimental period among
the groups administered ACR, ATRA, or vehicle control.
Moreover, abnormal blood chemistry such as liver dysfunc-
tion or hyperlipidemia, which is known to be associated with
overdose of vitamin A, was not seen (data not shown).
Therefore, our results were likely attributable to the primary
effects of the retinoid rather than secondary effects through
adverse effects.

ACR Induces Apoptosis in Neointimal Cells
We next examined whether the effect of ACR on inhibition of
neointima is attributable to ACR-induced apoptosis. For this,
we performed terminal deoxynucleotidyl transferase-
mediated dUTP nick end-labeling (TUNEL) staining on
cuff-injury samples. TUNEL index in the neointima was
significantly higher in the ACR-administered (100 mg/kg)
group than in the ATRA-administered group or the vehicle
control group. TUNEL index for the ATRA group showed
marginal values, and there was no significant difference
between the ATRA and vehicle control groups (Figure 2A
and 2B).

Next, to test whether ACR shows effects on cell prolif-
eration, we immunostained for proliferating cell nuclear antigen
(PCNA) as an established marker of cell proliferation.
Cotaining with TUNEL was not possible because of differ-
ence in reagents and procedure, but consecutive samples were
used. These studies showed that the PCNA index (PCNA
positive nuclei/total nuclei) was not affected by ACR admin-
istration (supplemental Figure IA and IB). These results
indicate that ACR induced apoptosis of neointimal SMCs in
vivo and may lead to inhibition of progression of neointima
formation by apoptosis and that effects on apoptosis rather
than effects on cell growth regulation was the main effect
of ACR in neointima formation. Therefore, the effect of inhibi-
tion of neointima formation correlated with the effect of ACR
on neointimal apoptosis.

Cellular Apoptosis Is Induced by ACR
We then confirmed the effect of ACR on cellular apoptosis in
vitro. First, SMCs were treated with ACR or ATRA and then
examined by TUNEL staining using confocal immunofluo-
rescence microscopy. Cells treated with ACR (1 μmol/L)
showed significant increase in TUNEL positive cells than in
cells with administration of vehicle (dimethylsulfoxide min-
imum [DMSO]; Figure 2C). The TUNEL index was consis-
tently higher in the groups administered ACR than for ATRA
or vehicle (DMSO) (Figure 2D). ACR thus induced cellular
apoptosis.

To next confirm the dose-dependency of apoptotic effects
of ACR on SMCs, cells were treated with ACR or ATRA
from $10^{-8}$ mol/L to $10^{-5}$ mol/L for 0 or 24 hours and analyzed
by DNA fragmentation assay. DNA fragmentation ratio for

Figure 2. A, TUNEL staining in cuffed artery speci-
mens obtained from mice at 35 days after cuff
placement. The arrows denote TUNEL-positive
cells. Scale bar=10 μm. B, TUNEL index (TUNEL-
positive cells/total number of cells) of ACR (100
mg/kg) and vehicle control (Cntl). **P<0.01. C, TUNEL
index of ACR (100 mg/kg) and vehicle control (Cntl). **P<0.01. D, TUNEL staining of rat SMCs treated
with ACR (10 μmol/L) or vehicle (DMSO, Cntl) for 24 hours. The right upper and lower panels show phase
contrast micrographs, and the left upper and lower panels show confocal-microscopic images. The
left and right panels show the same view. D, TUNEL index of ACR (10 μmol/L), ATRA (10 μmol/L), or vehicle (DMSO, Cntl). **P<0.01. E, DNA
fragmentation assay using rat SMCs cultured with
ACR or ATRA. The absorbance values represent-
ing DNA fragmentation at 0 hours were divided by
that at 24 hours. **P<0.01. F, Growth inhibition of
SMCs and endothelial cells (EC) under ACR or
ATRA treatment for 24 hours. The number of cells
treated with retinoids for 0 hours was considered
100%. Results were expressed as percentage of
growth with 100% representing untreated cells. Percentage of cells after retinoid treatment was
determined. The solid line (filled circles and open
rectangles) shows growth of SMCs, and the dotted
line (filled triangles and open diamonds) shows
growth of bovine aortic endothelial cells (BAECs). **P<0.01. All above experiments were reproduced
in at least 3 other occasions.
treatment with $10^{-7}$ mol/L of ACR was 1.5-fold higher than that of treatment by vehicle (DMSO) alone (Figure 2E, lanes 1 and 3). Additionally, that at $10^{-6}$ mol/L of ACR was 2-fold higher, and that at $10^{-5}$ mol/L of ACR was 3-fold higher as compared with treatment by vehicle (DMSO; lanes 1, 4, and 5). Furthermore, direct comparison of effects of ATRA and ACR on apoptosis by DNA fragmentation assay showed that ACR markedly induces apoptosis in a dose-dependent manner as compared with the dose-dependent yet very marginal increases as induced by ATRA (lanes 2 to 5). Therefore, ACR induced apoptosis of SMCs in a dose-dependent manner.

Furthermore, to compare effects between ACR and ATRA on growth inhibition, SMCs were treated with ACR or ATRA from $10^{-8}$ mol/L to $10^{-5}$ mol/L for 24 hours. Growth inhibition of SMCs treated with ACR $10^{-8}$ mol/L was greater than that treated with ATRA or vehicle (DMSO; Figure 2F), and was dose-dependent up to $10^{-5}$ mol/L (lanes 2 to 5). On the other hand, ACR mildly inhibited cell growth of endothelial cells, which was not significantly different from that when treated with ATRA (lanes 2 to 5). These results indicated that the growth inhibitory effect of ACR is cell-type dependent with preferential effects on SMCs. ACR, therefore, showed more potent effects on apoptotic SMCs growth inhibition as compared with ATRA.

**ACR Induces Expression of RARβ in SMCs**

ACR has been shown to preferentially act on RARβ and to elicit biological effects including apoptosis mainly through RARβ. RARβ, however, shows only marginal expression in SMCs. We therefore hypothesized that the effects of ACR on vascular cells may be mediated by effects on RARβ.

We examined effects of ACR on the expression levels of RARα, RARβ, and RARγ mRNA by RT-polymerase chain reaction (PCR) analysis and real-time PCR analysis. In samples treated with ACR, a marked increase in RARβ was seen starting at 6 hours after the addition of ACR which increased up to 12 and 24 hours. This effect was both dose-dependent and time-dependent, as $10^{-6}$ mol/L showed stronger effects as $1^{-5}$ mol/L, and as effects were more prominent after longer treatment. In contrast, no changes in RARα or RARγ were seen (Figure 3A, supplemental Figure IC to IE). Furthermore, RARβ was expressed in endothelial cells, but importantly, it was not induced by ACR as seen in SMCs (supplemental Figure IIA). ACR therefore induced expression of RARβ in SMCs.

We next compared effects of ACR and ATRA on RARβ expression. ATRA is known to marginally induce expression of RARβ in cultured SMCs. ACR induced RARβ expression in a more potent manner than ATRA in both dose-dependent and time-dependent manners at both the mRNA and protein levels (Figure 3B and 3C). ACR, therefore, potently induced expression of RARβ.

**ACR Increases Retinoic Acid Response Element (RARE)-Dependent Transcriptional Activity Through RARβ**

ATRA has been shown to induce endogenous expression of RARβ in cultured SMCs by acting on the retinoic acid...
responsive element (RARE) of the RARβ promoter. However, it remains to be clarified whether RARβ expression would also be affected by treatment of SMCs with retinoids other than ATRA, namely ACR. To next examine whether ACR preferentially regulates RAR-dependent transcription through RARβ, reporter assays were done using a reporter harboring a RARE or a retinoic acid non-responsive element (non-RARE [DR-1]) with expression of similar amounts of each RAR isoform and ATRA, ACR, or absence of ligand. Combination of ACR with RARβ transfection stimulated RARE reporter activity higher than with RARα or RARγ by approximately 2.2-fold (Figure 4A, lanes 6, 9, and 12). ACR did not stimulate the non-RARE (DR-1) promoter (supplemental Figure IIB). ACR therefore preferentially stimulates RARE-dependent transcription of the RARβ promoter. These findings, coupled with the fact that ACR selectively induces expression of RARβ in SMCs (Figure 3A to 3C), suggest that the biological effects of ACR in SMCs were mediated through RARβ.

RARβ Induces Apoptosis of Vascular SMCs

To determine whether the induction of apoptosis on SMCs by ACR is attributable to RARβ induction, we next examined whether RARβ induces apoptosis. DNA fragmentation assay in cells transfected with the RARs showed marginally increased DNA fragmentation ratio by approximately 1.5-fold for RARα and RARγ (Figure 4B, lanes 4 to 12). Importantly, cells transfected with RARβ showed a markedly greater dose-dependent increase by approximately 2.5-fold (lanes 8 and 9). These results show that expression of RARβ induces apoptosis in SMCs.

To confirm that RARβ mediates apoptosis, we used an RNA interference (RNAi) knockdown approach to examine the requirement of RARβ in mediating this response. Specific effects of the constructs and protocols as not to induce apoptosis were further established by use of the negative control construct against enhanced green fluorescent protein (EGFP). We first established conditions under which RARβ could be specifically knocked down by siRNA transfer. The specificity of the siRNA construct against RARβ was confirmed by lack of effect on related factors, RARα and RARγ (Figure 4C).

We next examined whether RARβ siRNA could inhibit apoptosis. siRNA was transfected into rat SMCs treated with ACR. After 24 hours incubation, cells were analyzed by DNA fragmentation assay. Under conditions in which ACR induced apoptosis, RARβ siRNA, but not control siRNA, inhibited apoptosis of SMCs treated with ACR in a dose-dependent manner (Figure 4D). These findings confirm that RARβ mediates cell growth as regulated by ACR.

ACR Increases RARβ Expression in Neointimal Cells

As the above results showed that ACR inhibits neointimal formation, that ACR is associated with increased apoptosis,
furthermore that ACR induces RARβ, and finally that RARβ induces apoptosis, then it would be expected that RARβ expression would be increased in the neointima of the cuff-injury model.

Immunohistochemistry of RARβ and double immunostaining of RARβ and SM α-actin (smooth muscle α-actin) to show that RARβ expressing cells are indeed SMCs were done using the cuffed artery samples treated with ACR (100 mg/kg) or vehicle control. We found that RARβ expression in the neointima was increased in the samples of the ACR-administered (100 mg/kg) group (supplemental Figure III). ACR therefore increased RARβ in the neointima of injured vessels.

Discussion

ACR Inhibits Neointima Formation by Inhibiting Cell Growth and Inducing Apoptosis

In the present study, we showed that ACR inhibits neointima formation after vascular injury. Of the retinoids, ATRA, in particular, has been shown to inhibit neointima formation in the rat balloon injury model.18,19 Our findings add to these previous studies by demonstrating that the retinoid derivative, ACR, also shows inhibitory effects on vascular remodeling through the cuff-injury model. Importantly, ACR has a unique high safety profile with lower frequency of adverse effects as compared with conventional retinoids, which is thought to be attributable to its unique acyclic structure.6 Interestingly, ACR also shows preventive effects on carcinogenesis (eg, recurrence) that distinguishes it from ATRA, which shows effects on tumor size reduction similar to ATRA.9 With promising expectations for therapeutic application of ACR because of this property, trials have been begun in liver cancer.5,9 Our present findings suggest that ACR may also show therapeutic (preventive) potential for vascular remodeling.

To understand the underlying effects of ACR in vascular remodeling, we investigated its cellular and biochemical mechanisms of action. ATRA has been shown to inhibit growth of SMCs and migration from the media to the intima, and to restore their phenotype in vascular lesions.20 We found that ACR can inhibit growth of SMCs similar to ATRA, but also that ACR induces apoptosis of neointimal cells. ATRA is known to induce cell cycle arrest,21 and also to induce differentiation of SMCs,22 but effects on apoptosis in vivo remain unclear. Likely a result of this additional pathway to induce apoptosis, ACR reduces neointima formation more potently than ATRA. These results suggest that exposure to ACR influences the response of vascular SMCs in vascular injury.

An important finding of the present study is that in contrast to most published studies on therapeutic intervention against neointimal formation which are centered on acute (first 24 hours) to subacute effects (up to 2 weeks) and thus leave in question their long-term therapeutic effects as would be necessary in the clinical setting, ACR uniquely was able to induce as well as maintain apoptosis of neointimal cells at least up to 5 weeks after injury, which suggests that it is a viable therapeutic agent for prolonged use. Our results therefore suggest that the mechanisms of action of ACR in inhibition of neointima formation are likely also attributable to inhibition of cell growth and stimulation of apoptosis.

RARβ Is a Molecular Target of ACR, and Upregulation of RARβ by ACR Leads to Apoptosis of SMCs

SMCs express all 3 RAR isoforms, but there is very little expression of RARβ.15 The role of RARβ in the vasculature is poorly known other than that ATRA can marginally induce RARβ in SMCs,15 ACR, however, has been shown to preferentially act on RARβ.7,8 We therefore sought to understand the role of RARβ in vascular remodeling. Our results suggest ACR may exert inhibitory effects on vascular remodeling at least in part through upregulation of RARβ in SMCs. Increased inducibility of RARβ in SMCs may be a critical determinant of increased apoptosis by ACR as compared with ATRA.

There are a number of cells which show endogenous expression of RARβ (eg, endothelial cells, esophageal cells)23,24 where its physiological function seems to be regulation of senescence,25 but it seems that induction of RARβ in nonexpressed cells, such as in cancer cells (eg, lung carcinoma cells)25 in addition to SMCs as shown in the present article, has been shown to induce apoptosis which is consistent with its role as a potential tumor suppressor. One report which addressed the acute apoptotic effects of vascular injury (eg, less than 4 hours after injury) documented decreased expression of the apoptotic regulator, bcl-X, not only in the neointima but also in the luminal region of the media.26 This modulation of bcl-X expression was coupled with increased TUNEL-positive nuclei and thus apoptosis in medial SMCs on the luminal side.26 The different effects on luminal SMCs was thought to be attributable to increased mechanical stress on the luminal side.26 In the present study, medial SMCs showed a different response as compared with neointimal SMCs in terms of the functional expression of RARβ. That is, in contrast to neointimal SMCs which showed induction of RARβ which was associated with apoptosis, medial SMCs showed greater expression on the adventitial side as compared with the luminal side, but did not manifest apoptosis. We envision that mechanical stress on the luminal side affected expression of RARβ within the media. However, we do note that the medial area was not decreased nor showed apoptosis despite expression of RARβ, which is consistent with previous reports which have documented the lack of medial SMC apoptosis at later stages.27,28 Further investigation will be necessary to understand possible cell-type specific effects (ie, medial SMCs being more resistant to apoptosis) as well as stage-specific effects (ie, late as compared with acute effects) in addition to the physiological and pathological roles of RARβ in the vasculature.

To our knowledge, very little is known of the molecular actions of ACR on apoptosis other than an observation that ACR induces apoptosis of hepatoma cells through tissue transglutaminase.9 Further, the molecular actions of RARβ on apoptosis have also been poorly addressed, other than a single report on apoptotic activity in oral cancer cells.29 ACR has also been shown to selectively act on RARβ in hepatoma...
cells. It is noteworthy that tissue transglutaminase seems to be a common downstream proapoptotic pathway for ACR and ATRA, although the precise role of ATRA in apoptosis still remains controversial. Apoptotic effects are presently thought to be selective for synthetic and not natural retinoids and to be ascribed to intracellular target proteins which are independent of nuclear receptor functions. Further, both ACR and ATRA can induce RARβ through RARE-dependent transcription. It is therefore difficult to clearly delineate a specific pathway whereby the apoptotic actions of ACR are selectively mediated through RARβ, given the presence of crosstalk as well as likely hitherto unknown independent pathways, but as a similar pathway of ACR mediated apoptosis through RARβ has been documented in hepatoma cells, it is tempting to speculate that this common pathway dominantly functions to selectively induce apoptosis at least in these cell types including neointima cells and hepatoma cells.

In summary, ACR leads to growth inhibition and apoptotic induction in SMCs. Additionally, ACR, which exerts its actions through RARβ, induces RARβ which in turn induces apoptosis in SMCs. This regulatory pathway through activation of the RARβ pathway by pharmaceutical intervention may be potentially exploitable for therapeutic purposes. Taken together, ACR may be a promising agent for therapeutically inhibiting neointima formation.

Disclosures

None.

References

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MATERIALS AND METHODS

Chemicals

ACR (3,7,11,15-tetramethyl-2,4,6,10,14,-hexadecapentaenoic acid) was provided by Nikken Chemical Co., and ATRA and dimethyl sulfoxide minimum (DMSO) were purchased from Sigma-Aldrich (Table 1). ACR and ATRA were dissolved in DMSO.

In situ Apoptosis Detection (TUNEL method)

The in situ TUNEL detection kit (TaKaRa) was used. Sections of cuffed arteries from formalin fixed and paraffin-embedded blocks were deparaffinized and rehydrated using the manufacturer’s suggested method. Samples (N=18, 6 from the vehicle control group, 6 from the ATRA group and 6 from the ACR low group) were permeabilized with proteinase K and terminal deoxynucleotidyl transferase (TdT) was added. The immunoreactive positive cells were visualized with diaminobenzidine tetrahydrochloride (DAB) (Dako) then counterstained with hematoxylin (Wako). TUNEL-positive nuclei were assessed by the TUNEL index, which was calculated as the ratio of TUNEL-positive nuclei to total nuclei as described.\(^1\) Approximately 200 cells were counted with reproducible results. Experiments were reproduced in at least three other occasions.

Cell culture

Rat SMCs were isolated as described.\(^2\) Cells were cultured in DMEM/F12 (Invitrogen) supplemented with 10\% fetal bovine serum (FBS) (Hyclone laboratory),
100 U/ml penicillin and 100 µg/ml streptomycin. C2/2 cells, which were derived from
the rabbit thoracic aorta and released by collagenase treatment as described,\textsuperscript{3} were
grown in DMEM (Sigma-Aldrich) supplemented with 10% FBS, 100 U/ml penicillin
and 100 µg/ml streptomycin. BAECs (Bovine Aortic Endothelial Cells), initially
isolated from the descending bovine aorta by mechanical scraping, have been
previously described.\textsuperscript{4} Cells were grown in DMEM supplemented with 10% FBS, 100
U/ml penicillin and 100 µg/ml streptomycin. HUVECs (Human umbilical endothelial
cells) (Cambrex) were grown using EGM\textsuperscript{TM}-2 SingleQuots (Cambrex) following the
manufacturer’s instructions.

**Apoptosis analysis**

For morphological analysis, rat SMCs were treated with 10\textsuperscript{-6} mol/L ACR,
ATRA or DMSO (as vehicle control) for 24 hours, then trypsinized and washed with
PBS. After fixation with paraformaldehyde followed by acid-alcohol treatment, cells
were incubated with TdT using the \textit{in situ} TUNEL detection kit (TaKaRa). Stained
cells were examined with a fluorescent confocal microscope. Experiments were
reproduced in at least three other occasions.

**DNA fragmentation assay**

Rat SMCs treated with ACR or DMSO (as vehicle control), and C2/2 cells
were transfected with pRS-RSV empty vector or pRS-RAR\textalpha, pRS-RAR\textbeta or
pRS-RAR\textgamma expression vectors using Tfx-20 (Promega) following the manufacturer’s
instructions. After 0 or 24 hours, cells were harvested and then apoptosis was detected
by DNA fragmentation assay using the Cell Death Detection ELIZA\textsuperscript{PLUS} Kit (Roche)
according to the manufacturer’s instructions. Whole cell lystate of C2/2 cells overexpressing the RAR isoforms individually, was then analyzed by Western blot using anti-RARα (Santa Cruz), anti-RARβ (Santa Cruz), anti-RARγ (Santa Cruz) or anti-GAPDH (Ambion) antibodies. The assay was done in duplicate and reproduced in at least three other occasions.

Cell count analysis

Rat SMCs and BAECs were plated on 60 mm-plastic wells (1x10⁵ cells/well) at cell density of 60%-70% confluency. They were treated for 24 hours in medium containing 1% FBS supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin with the indicated concentrations of ACR, ATRA or DMSO (as vehicle control). Cell numbers were counted by the trypan blue dye exclusion method. To examine the growth-inhibitory effects of ACR, ATRA or DMSO (as vehicle control), the cells were treated for 24 hours. Approximately 200 cells were counted. Experiments were reproduced in at least three other occasions.

RT-PCR analysis

Rat SMCs and HUVECs were treated with ACR, ATRA or DMSO (as vehicle control) for 6, 12 or 24 hours. They were then harvested and total RNA was isolated by the RNeasy preparation kit (Qiagen). cDNA was amplified from 5 µg of total RNA. Quantitative PCR was done with a gene-specific primer and a Quantum RNA 18S internal standard primer set (Ambion) as described previously. The sequences of primers for RARα and RARβ were previously reported. Primers used for amplification were as follows;
RARα, 5’-CAGATGCACAACGCTGGC-3’ and 5’-CCGACTGTCCGCTTAGAG-3’,
RARβ, 5’-ATGCTGGCTTCGGTCCTC-3’ and 5’-CTGCAGCAGTGGTGACTG-3’,
and RARγ, 5’-GTGGAGACCGAATGGACC-3’ and
5’-CCCTGGAGCTTCATCCTC-3’. After PCR amplification, the fragments were
analyzed by agarose gel electrophoresis. Experiments were reproduced in at least three
other occasions.

Real-time PCR was performed using LightCycler (Roche) and QuantiTect
SYBR green PCR kits (Qiagen) following the manufacturers’ instructions.

Western blot analysis

Rat SMCs were treated for 12 and 24 hours with the indicated concentrations
of ACR, ATRA or DMSO (as vehicle control). They were then washed and suspended
in lysis buffer (Promega). The suspended cells were harvested and normalized to
protein concentration according to the Bradford method (Bio-Rad). Western blot
analysis was then performed using anti-RARβ (Santa Cruz) or anti-GAPDH (Ambion)
antibodies. Experiments were reproduced in at least three other occasions.

Co-transfection reporter assay

5x10⁴ C2/2 cells were plated into a 24-well plate. After 24 hours,
RAR-responsive element (RARE) reporter (DR5-luci), RAR non-responsive element
(non-RARE (DR-1)) reporter (DR1-luci),⁷ and pRS-RSV empty vector or pRS-RARα,
pRS-RARβ or pRS-RARγ expression vector was transfected by lipofectamine 2000
(Invitrogen). pCMV β-gal was co-transfected for normalization of efficiency. After
transfection, the C2/2 cells were treated with ACR (1 μmol/L), ATRA (1 μmol/L) or
DMSO and incubated for 48 hours, then subjected to luciferase assay (Promega) by luminometry (Lumat LB9507, Berthold). β-Galactosidase activity was measured according to the manufacturer’s instructions (Promega). Assays were done in duplicate and reproduced in at least three other occasions.

**Immunohistochemistry**

The segments of the cuffed artery were deparaffinated and rehydrated. After washing, samples were treated with 3% peroxidase blocking reagent (Dako) to reduce endogenous peroxidase activity. After that, the samples were incubated with blocking goat serum (Sigma-Aldrich) and subsequently incubated with mouse-IgG (Santa Cruz) or anti-RARβ (Santa Cruz) antibodies at 4°C (N=12, 6 from the vehicle control group and 6 from the ACR low group) or anti-PCNA (proliferating cell nuclear antigen) (Dako) antibodies at 4°C (N=10, 5 from the vehicle control group and 5 from the ACR low group) or anti-SM α-actin (smooth muscle α-actin) (Sigma-Aldrich) at 25°C (N=20, 5 from the vehicle control group, 5 from the ATRA group, 5 from the ACR low group and 5 from the ACR high group). After washing, the samples were treated using the DAKO ENVISION kit/HRP (Dako) following the manufacturer’s instructions. Samples were counterstained with hematoxilin (Wako). Double immunostaining using anti-RARβ (horse radish peroxidase: brown) and anti-SM α-actin (alkaline phosphatase: red) antibodies visualized by diaminobenzidine (DAB) and fuchsin, respectively, was performed according to the manufacturer’s protocol (Dako) (N=12, 6 from the vehicle control group and 6 from the ACR (100 mg/kg) group). Experiments were reproduced in at least three other occasions.
RNA interference (siRNA)

siRNA constructs were made according to the manufacturer’s protocol for the Silencer™ siRNA Construction Kit (Ambion).

The siRNA oligonucleotide probes were as follows.

RARβ sense : AAGGATGGTGCCGGAATAAACCCTGTCTC (29mer)
RARβ antisense : AAGTTTATTCCGGCACCATCCCCCTGTCTC (29mer)
EGFP (enhanced green fluorescent protein) (control) sense:
AAGTCGTGCTGCTTACATGTGGCGCTCTC (29mer)
EGFP (control) antisense: AACCACATGAAGCAGCACGACCCTGTCTC (29mer)

10, 50 or 100 pmol/L of double-stranded 29-mer siRNA constructs were transfected into rat SMCs after ACR treatment for 24 hours using lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. After 0 or 24 hours incubation, cells were analyzed by DNA fragmentation assay. Experiments were reproduced in at least three other occasions.

Statistical analysis

All data were expressed as mean value ± standard deviation. Statistical analysis for cuff injury experiments was done by the Mann-Whitney U test, of the DNA fragmentation assays by either Student’s t-test or Welch’s t-test, and of the cotransfection reporter assays by ANOVA as corrected by the Bonferroni method. P<0.05 was considered statistically significant.
REFERENCES


6. Wan YJ, Wang L, Wu TC. Expression of retinoic acid receptor genes in

FIGURE LEGENDS

Figure I.  A, PCNA staining in cuffed artery specimens obtained from mice at 35 days after cuff placement. The arrows denote PCNA-positive cells. Scale bars 10 µm.  B, PCNA index (PCNA positive nuclei/ total nuclei) of ACR (100 mg/kg) and vehicle control (Cntl). Approximately 200 cells were counted with reproducible results. **, p<0.01.  C-E, Temporal profile of mRNA expression of RAR isoforms in response to ACR treatment from 1 µmol/L to 10 µmol/L as assessed by real-time RT-PCR. The number of transcripts for each gene was normalized to that of the internal control, 18s rRNA. Normalized values were further compared with samples obtained from cells treated with vehicle (DMSO, Cntl) at 6 hours.  C, Relative mRNA expression of RARα. D, Relative mRNA expression of RARβ. E, Relative mRNA expression of RARγ.

Figure II.  A, Temporal profile of mRNA expression of RARβ in response to ACR or ATRA administration from 1 µmol/L to 10 µmol/L in endothelial cells (HUVECs). 18S was used as internal control. Note that ACR did not influence the mRNA levels of RARβ in endothelial cells. M: Marker. B, non-RARE (DR-1) promoter activity transfected with empty or RAR isoforms under ACR (1 µmol/L) or ATRA (1 µmol/L) administration and in the absence of ligand in C2/2 cells. Note that ACR did not activate non-RARE (DR-1) promoter activity in cells expressing RAR isoforms.

Figure III. Immunohistochemistry for RARβ and double-immunohistochemistry for RARβ and SM α-actin in cuffed artery samples obtained from mice. Low-powered views (a, b, e, f) and high-powered views (c, d, g, h) are shown. The arrows denote
RARβ-positive cells in c and d. In g and h, RARβ-positive cells are shown in brown (arrows) and SM α-actin-positive cells are shown in red (arrowheads). Scale bars 50 μm (a, b), 25 μm (e, f), 10 μm (c, d, g, h). Immunostaining for RARβ was reproduced in at least three other occasions and double-immunostaining was reproduced in at least two other occasions.
Table 1. Structure of ACR and ATRA.

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ACR : (2E, 4E, 6E, 10E)-3, 7, 11, 15-tetramethyl-
2, 4, 6, 10, 14,-hexadecapentanoic acid

ATRA : all-trans retinoic acid
Fig. I - Kada et al.
**A**

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**Fig. II - Kada et al.**
Fig. III - Kada et al.