Retinoic acid upregulates β₁-integrin in vascular smooth muscle cells and alters adhesion to fibronectin

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Retinoic acid upregulates β₁-integrin in vascular smooth muscle cells and alters adhesion to fibronectin. Am J Physiol Heart Circ Physiol 279: H382–H387, 2000.—Retinoic acid has an established physiological role in differentiation, development, and cellular growth. This study investigated the action of all-trans retinoic acid (ATRA) on vascular integrins, cell-surface receptors that control growth and remodeling of blood vessels. The β₁-integrin subunit mRNA and protein was induced after treatment with ATRA in two different rat vascular smooth muscle cell lines. To relate this result to the in vivo state, the aortas from adult rats fed with therapeutic doses of ATRA were examined for β₁-integrin protein. A significant upregulation of the integrin subunit was observed in vivo. To assess if this increase contributed to physiological changes in cellular function, cells treated with ATRA were tested for alterations in adhesion to extracellular matrix proteins. The cells exposed to the retinoid were seen to adhere more strongly to fibronectin, via the β₁-integrin. These results showed that modulation of vascular integrins by ATRA in adult rats contributes to functional changes that can cause remodeling of blood vessels.

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**METHODS**

**Cell culture.** Rat aortic smooth muscle cells (RASMCs; see Ref. 21) and a pulmonary arterial smooth muscle cell line (PAC; see Ref. 26) were obtained from Dr. Joe Miano and were cultured as mentioned in each protocol below. ATRA (Sigma Chemical, St. Louis, MO) and cells being treated with it, were always handled in the dark to avoid photoactivation as described previously (20, 21).

**Northern analysis.** RASMC and PAC were grown to 80% confluency in DMEM containing 10% FBS and antibiotics, washed one time with DMEM, and treated overnight with 1 μM ATRA or the same volume of vehicle (DMSO) in DMEM-F-12 media (Sigma) containing 0.1% BSA. Total RNA was extracted from the cells using the Trizol reagent (3 ml/100-mm dish; GIBCO-BRL, Gaithersburg, MD), as specified by the manufacturer. Equal amounts of RNA from each sample (20 μg) were loaded on a formaldehyde agarose gel and were electrophoresed, blotted, and probed with β1-cDNA as described (16). The β1-probe was isolated after RT-PCR from the PAC total RNA (28) using the RT-PCR Ready-To-Go-Beads (Pharmacia, Piscataway, NJ) with β1-specific oligomers 5′-ccagccatccagctgcatg-3′ and 5′-tccacgcatcagcgtgggcaac-3′. The product was electrophoresed on a 1% agarose gel, and a single band of expected size (644 bp) was purified with Eluquick beads (Schleicher and Schuell, Keene, NH). It was confirmed to be a β1-integrin product by Southern analysis (28) using the labeled β1-specific internal oligomer 5′-agagaaacgctgatg-3′ as a probe. The β1-specific PCR product hybridized to the probe, whereas other DNA fragments on the same membrane did not.

This fragment was used as a cDNA probe by labeling 25 ng with [32P]dCTP (3,000 Ci/mmol; NEN Life Science Products, Boston, MA) using Ready-To-Go dCTP DNA-labeling beads (Pharmacia). The labeled product was separated from unincorporated nucleotides using Microspin S-200 HR columns (Pharmacia) and was used for hybridization as described (16).

The ethidium bromide-stained gels and autoradiographs were scanned in a VISTRA fluorimager and densitometer, respectively. Quantitative values for the 28S rRNA in each lane were obtained and used to normalize the corresponding smooth muscle β1-mRNA band. The experiments were independently repeated three times for the RASMC and four times for the PAC samples.

**Western analysis.** RASMCs and PACs were cultured for 10 days in 100-mm dishes with vehicle (DMSO) or ATRA (1 μM). The serum was reduced to 0.5% after the cells reached 50% confluency. Hormones and media were replaced every 48 h. The suspension was administered to 8-wk-old male Sprague-Dawley rats orally using an 18-gauge gavage needle. Four rats were administered 1 mg/kg RA every other day for 3 wk. At the same time, a similar number of animals (controls) was given corn oil alone. The rats were weighed throughout the experimental period, and the amount of ATRA was adjusted for changes in body weight.

At the end of the 3 wk, the animals were killed, and the aortas were harvested. A similar length of tissue from the same stretch of the aorta was taken from each animal and homogenized by hand in RIPA buffer. The total protein in the lysates was estimated, and equal amounts (45 μg) from each sample were used for a Western blot to evaluate the β1-integrin and smooth muscle α-actin, as already described above for the cell lines.

**Statistical analysis.** All statistical analyses were performed between ATRA-treated samples vs. vehicle-treated control groups using SigmaStat 2.0 software. For antibody blocking in the adhesion assays, the tests were also run between samples treated with blocking antibodies or controls treated with class-matched immunoglobulins. Analyses were performed.
presented as unpaired mean values ± SE in two-tailed, one-way ANOVA tests. Tukey tests were run after the ANOVA to determine the significance of the difference between the variability of the mean values between the two groups. All data reported were statistically significant (P < 0.05 or as specifically mentioned in each result) and satisfied the Tukey test requirements.

RESULTS

Effects of ATRA on expression of β1-mRNA. Integrin profiles on vascular smooth muscle cells are very important to determine their fate. It has been shown that ATRA substantially attenuated the proliferative action of platelet-derived growth factor (PDGF-BB) and serum stimulation in VSMCs in culture (21). The present study attempts to correlate integrin expression with growth inhibitory doses of ATRA in the same RASMCs. The status of the RA receptors in these cells has already been characterized (21). To further verify the results, a second line (PACs; see Ref. 26) was also studied. Both lines were grown in culture and treated for 24 h with vehicle (DMSO) or ATRA (1 μM) dissolved in DMSO. The total RNA was extracted, electrophoresed as described in METHODS, and probed with labeled β1-integrin cDNA. The samples showed only one band hybridizing to the probe at ~4.6 kb (Fig. 1A), which is the same size already described for the rat β1-integrin (4). The amount of RNA loaded in each lane was normalized by quantitating the ethidium bromide-stained 28S or 18S bands in a fluorimager, and this value was used to express the amount of specific β1-mRNA in each lane. There was a significant increase in the β1-message in both cell lines after treatment with 1 μM ATRA (P = 0.02 for n = 3 in RASMC, P = 0.0021 for n = 4 in the PACs), as shown in Fig. 1B. The steady-state message was increased >60% after only 24 h of treatment with ATRA at concentrations that affected PDGF-BB or serum-stimulated growth of these cells.

Total β1-protein expression in VSMC. It was more difficult to see a specific increase in β1-protein after treatment with ATRA, as integrin messages are often long-lived and abundant (16). Also, regulation of these messages at the translational level remain poorly understood. In an effort to document the change in β1-protein, the cells were treated with ATRA for varying times. An increase in expression was seen after 3 days of exposure to ATRA and was very significantly up-regulated after 10 days (Fig. 2A). The delay to observe

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**Fig. 1.** Northern hybridization of vascular smooth muscle cell (VSMC) RNA after treatment with all-trans retinoic acid (ATRA). **A:** blot showing the β1-specific mRNA (~4.6 kb, top) in control cells (C, treated with vehicle) and cells treated with ATRA (R, 1 μM). **Bottom:** ethidium bromide-stained 28S rRNA. RASMC, rat aortic VSMC; PAC, pulmonary artery VSMC. **B:** graph showing the quantitative increase in β1-integrin message after treatment with 1 μM ATRA for 24 h. Each value for β1-mRNA was normalized for amount of RNA loaded on the gel using the corresponding densitometric reading for the 28S rRNA. n, No. of preparations.

**Fig. 2.** Western blots showing increase in total β1 expressed in RASMCs and PACs after treatment with retinoic acid (RA). **A:** proteins from 10-day ATRA (1 μM)-treated cells (R) and controls (C) were used for Western analysis. Each gel was probed sequentially with 2 antibodies; one antibody was for the β1-integrin protein (~121 kDa when nonreduced; top), and the second was a smooth muscle specific α-actin monoclonal antibody (bottom; demonstrating equal loading in each lane). **B:** quantitative changes of total β1-integrin protein in RASMCs and PACs after treatment with ATRA, after normalization with α-actin.
early changes in protein may also be due to technical factors such as affinity of the antibody and sensitivity of chemiluminescent assay used for this protocol. This is in contrast to Northern blots where hybridization of mRNA to long (>100 bp) [32P]DNA is very sensitive. The nonreduced β1-protein was around 121 kDa as has been published (18). We confirmed the integrity of this band with a second anti-CD29 polyclonal antibody (AB 1937; Chemicon; results not shown). The β1-bands from independent experiments were quantitated and normalized for protein loading from the corresponding densitometer reading of the smooth muscle α-actin bands in each sample. Treatment with ATRA increased the expression of the β1-integrin subunit in both cell lines, as seen in Fig. 2, A and B, with \( P = 0.01 \) for \( n = 4 \) in RASMCs and \( P = 0.001 \) for \( n = 3 \) in PACs. The upregulation in the β1-protein was most likely more pronounced than the increase in the total β1 message seen in Effect of ATRA on expression of β1 mRNA due to the longer treatment with ATRA.

Effect of RA on β1-integrin in vivo. To see if this increase in β1-integrin expression would occur in vivo, 8-wk-old adult male rats were given oral doses of ATRA for a prolonged period (3 wk). Previous studies (20) have reported that 2 wk and 4 days of treatment with ATRA had significant effects on vascular remodeling after balloon withdrawal injury of the common carotid artery in rats. The 3-wk time point chosen for this study was to ensure that uninjured aortas were given ample chance to express changes in protein induced by the treatment. The dose selected was close to that administered for therapy to patients with APL and was less than the dose fatal to 50% of test animals for ATRA. After 3 wk of treatment, the rats were euthanized, and their aortas were examined for β1-protein expression. As shown in Fig. 3A and as seen in the case of the cultured cell lines, there was a consistent increase in β1-expression in the aortas of the ATRA-treated animals (\( P = 0.002, n = 4 \)). We were not able to quantitatively determine the class of cells that contributed to this result, although the VSMCs are the best candidates for this since they are the most abundant cell type in the aortas and have shown growth regulation by ATRA (20). Examination of another vessel, the basilar artery, did not give such reproducible results. Closer analysis of this result showed that the ratio of smooth muscle α-actin to total protein in the basilar arteries decreased after treatment with ATRA (unpublished observation), implying that RA could initiate other remodeling events in this vessel.

**Effects of ATRA on cell adhesion.** The cellular consequences of induction of β1-integrin by ATRA were analyzed using cultured VSMCs. Alteration of the adhesive profile of the cells that change interaction with the matrix would be signaled to the interior of the cell. RA may act by inducing adhesive changes that trigger antiproliferative pathways and stimulate cellular differentiation. To test this, the cultured RASMCs and PACs were treated with ATRA (1 μM) for 5 days and then were used to assay changes in cellular adhesion. Treatment beyond this time (10 days, as used to assay for upregulation of β1-protein) was not possible as it was difficult to lift the cells off the dish nonenzymatically due to the prolonged accumulation of secreted matrix. Harsh enzymatic digestion would likely destroy the extracellular integrin receptors. The cells

*Fig. 3. Western analysis of rat aortic β1-integrin protein after prolonged feeding of ATRA (1 mg · kg \(^{-1} \) · day \(^{-1} \)) for 3 wk. A: proteins of corresponding segments of the aorta from 4 control (C1–C4) rats and 4 ATRA-fed littermates (R1–R4) were probed sequentially with β1 and then smooth muscle (SM) α-actin monoclonal antibodies. B: values for β1-integrin normalized with the smooth muscle α-actin.*

*Fig. 4. Effect of ATRA on VSMC adhesion to fibronectin (5 μg/ml). The two cultured cell lines RASMC and PAC were treated for 5 days with RA and used for analyses for adhesion to fibronectin. Values for nonspecific adhesion to uncoated wells were subtracted to obtain results for specific binding only. There was a >3-fold increase in adhesion of the RASMCs, which was not significantly altered by teaching the cells with nonspecific hamster IgM. However, pretreating the cells with β1-specific antibody blocked this ATRA-induced increase in adhesion, showing that it was brought about by β1-integrins. This increased adhesion was not mediated by β3-integrins on PACs, as shown by using ascites fluid (IgAF) or anti-β1-monoclonal F-11.*
were plated on a number of substrates, including vitronectin, laminin, collagen, fibrinogen, and fibronectin. The only significant change in adhesion in cells treated with ATRA was seen in the case of fibronectin. This is demonstrated in Fig. 4 in which both cell types (RASMC and PAC) showed increased adhesion and spreading on fibronectin after treatment with ATRA ($P = 0.0001$ for $n = 10$ for RASMCs and $P < 0.0001$ for $n = 10$ in PACs). The untreated RASMCs adhered better to fibronectin than the PACs, but the increase in adhesion after exposure to ATRA was greater for the PACs. This could be due to a different array of $\alpha$-subunits that heterodimerize with the $\beta_1$-integrins being induced by ATRA within the two cell lines. Particularly interesting was the fact that the change of adhesion to fibronectin after exposing the RA-induced cells was completely blocked by the functional $\beta_1$-antibody Ha2/5 (18 and Fig. 4; $P = 0.008$ and $n = 3$ for RASMCs and $P = 0.0011$ and $n = 3$ for PACs). This was not seen when nonspecific hamster IgM (negative control) or anti-rat $\beta_3$-blocking antibody (F-11; see Ref. 13) and matching purified ascites fluid were used in PACs in a separate experiment (see Fig. 4). The blocking anti-$\beta_3$-monoclonal and ascites fluid (control) did not block adhesion, but both treatments increased nonspecific binding of RASMC to fibronectin and were not included in Fig. 4. The experiment thus clearly demonstrates that the upregulation in expression of the $\beta_1$-integrin subunit by ATRA was responsible for the altered adhesion of the two cell lines to fibronectin.

**DISCUSSION**

The aim of this study was to test the hypothesis that RA-induced $\beta_1$-integrin expression and altered VSMC adhesion, events that could result in inhibition of growth and initiation of tissue remodeling. RA has been shown to antagonize the growth-promoting actions of PDGF-BB and serum in cultured RASMCs (21). The results obtained in this study clearly show that similar concentrations of ATRA induce the expression of the $\beta_1$-integrin subunit in these cells. The increase was observed in the steady-state message and protein levels in both RASMC and PACs. There are a number of examples where $\beta_1$-integrins have been associated with decreased cell proliferation (10, 19, 24). Furthermore, there is evidence that decreased proliferation of chronic myelogenous leukemia progenitors and K-562 cells was mediated by a $\beta_1$-integrin, which resulted in increased adhesion to fibronectin (15).

Treatment of RASMCs and PACs with ATRA also showed an increased adhesion to fibronectin. Fibronectin can bind to a number of integrins, e.g., $\alpha_5\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_3$, and $\alpha_6\beta_6$ (27). The results from the present study showed that the RA-induced adhesion to fibronectin was completely blocked by a specific $\beta_1$-integrin functional antibody demonstrating it is mediated by $\beta_1$-integrins only. There was no change in RA-induced adhesion to fibronectin when an antibody to rat $\beta_3$-integrin, F-11, (unpublished observation) was used, confirming that ATRA increased cellular interaction with fibronectin in VSMC via a $\beta_1$- and not a $\beta_3$-integrin. The $\beta_3$-integrins have been seen to promote vascular proliferation (3, 9). It is therefore entirely reasonable to expect the antigrowth effect of the retinoids to be mediated by an increase in the $\beta_1$-integrin expression, which also enhances adhesion to fibronectin.

There were a number of reasons to check if the increase in $\beta_1$-integrin observed in cultured cells also occurred in vivo. First was the preliminary step to verify the observations recorded in cell lines as being relevant in living animals. Second, ATRA has been seen to induce remodeling of the carotid artery after balloon injury, reducing intimal hyperplasia and increasing vessel wall perimeter (20). Both events increase the lumen size, favoring the use of retinoids as therapeutic agents for prevention of restenosis. Third, chronic doses of the retinoids are being administered to patients with APL that could result in remodeling of their vasculature. For the last two reasons, the findings in this study that $\beta_1$-integrins are upregulated by ATRA merit closer and more detailed investigations.

The $\beta_1$-integrins have been described as key molecules in remodeling. In VSMCs, dynamic conformational changes in $\beta_1$-integrins were necessary for collagen matrix reorganization (14). The $\alpha_1\beta_1$ is a critical receptor in rat artery smooth muscle cells involved in matrix remodeling after injury (12). With the use of a mouse cell line lacking $\beta_1$-integrin, $\alpha_5\beta_{1A}$ was found to be a prime function for fibronectin matrix assembly (31). Therapeutic doses of ATRA were therefore used in this study to quantitate any changes in $\beta_1$-integrins in the blood vessels of adult rats. The results demonstrated a clear increase in the total $\beta_1$-integrin protein in the aorta, validating the observations made in VSMCs in culture. It was surprising to see variable amounts of $\beta_1$-integrin in a smaller vessel, the basilar artery, where the drug seemed to induce more dramatic remodeling. The specific VSMC marker smooth muscle $\alpha$-actin seemed to diminish with the treatment, implying that these cells were dying out. This complicated efforts to record reproducible changes in $\beta_1$-integrin (especially an increase) in these vessels after treatment with ATRA. This event did not seem to occur in uninjured carotid arteries (20). Thus there is a need to assess the effects of ATRA on remodeling of different vascular beds and vessel sizes in adults, to assess current treatment for patients with APL, and to benefit pathological procedures such as intimal disease associated with angioplasty and hypertension.

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