All-trans retinoic acid regulates proliferation, migration, differentiation, and extracellular matrix turnover of human arterial smooth muscle cells

Dorothea I. Axel*a, Anatol Friggea, Jasmin Dittmanna, Heike Rungea, Ioakim Spyridopoulosb, Reimer Riessena, Richard Viebahnb, Karl R. Karschc

*Medical Clinic III, Department of Cardiology, University of Tübingen, Otfried-Müller St. 10, D-72076 Tübingen, Germany
bDepartment of Surgery, University of Tübingen, Tübingen, Germany
cBristol Heart Institute, University of Bristol, Bristol, UK

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Abstract

Objective: The vitamin-A derivative all-trans retinoic acid (atRA) is a potent regulator of cell growth, differentiation, and matrix formation of various cell types and plays an important role in embryogenesis. However, sparse data are available about its effects on human vessel diseases. Thus, we studied the effects of atRA on human arterial smooth muscle cell (haSMC) and endothelial cell (haEC) proliferation, migration, differentiation and extracellular matrix (ECM) turnover in mono- and transfilter cocultures.

Methods: Effects of atRA on human arterial cells in monocultures were determined using cell counting assays, BrdU-ELISA and MTT-tests. In transfilter cocultures haSMC-growth was studied under the stimulatory effect of proliferating haEC. Using Northern blot analysis, effects of atRA on mRNA expression of ECM-proteins were examined while protein expression and activity of matrix metalloproteinases were determined by Western blotting and zymography.

Results: atRA caused a dose dependent inhibition of haSMC-growth in monocultures (IC50 at 0.022 μM) whereas haEC-growth was inhibited less potently (IC50 at 97 μM). In addition, proliferation and migration of haSMC through a50 porous membrane were inhibited dose dependently by micromolar atRA-doses after non-stop and single dose application of atRA on the endothelial side of the complex transfilter coculture system. Immunostainings and Northern blotting demonstrated an enhanced α-smooth muscle actin and heavy chain myosin expression in haSMC after atRA-treatment. Whereas mRNA-expression of the glycoproteins thrombospondin-1 and fibronectin were decreased, collagen-1 mRNA expression was even slightly stimulated. Transcription of biglycan and TGF-β1 were not influenced in a specific manner. Finally, protein expression and activity of the matrix metalloproteinases MMP-2 and MMP-9 were inhibited significantly by atRA. Conclusions: atRA was found to be a potent inhibitor of both haSMC-proliferation and migration, even in coculture with haEC releasing growth factors. In addition, redifferentiation, ECM synthesis and ECM degradation were regulated by atRA which also influence haSMC migration and intima formation. Thus, atRA-treatment seems to be a promising strategy for the inhibition of processes involved both in atherosclerosis and restenosis.

Keywords: Cell culture/isolation; Cell communication; Extracellular matrix; Smooth muscle; Restenosis

1. Introduction

Despite numerous in vitro and in vivo studies with a great variety of pharmacological compounds exerting growth inhibitory effects on smooth muscle cells (SMC) [1–4], the problem of restenosis after percutaneous interventions is still unsolved [3]. Although very potent anti-mitotic agents, such as paclitaxel (Taxol®), have been used showing very efficient inhibitory effects on smooth muscle cells in vitro [4] and in animal studies using local drug devices [5], efficacy in human coronary arteries has not been shown so far [6]. Beside proliferation of smooth muscle cells, migration, phenotypic differentiation, as well as extracellular matrix (ECM) formation and degradation

*Corresponding author. Tel.: +49-7071-298-7338; fax: +49-7071-295-749.
E-mail address: daaxel@med.uni-tuebingen.de (D.I. Axel).
determine the extent of intimal thickenings [7]. The predominant feature of late restenotic lesions is a large amount of extracellular matrix with a reduced number of smooth muscle cells, whereas in the early stages of intimal thickening formation the number of SMC is increased [8]. To successfully interfere with and modulate these processes, compounds are necessary which exert multifactorial effects on cellular activation and expression of extracellular matrix constituents. Thus, a single antiproliferative approach for the prevention of restenosis, as performed in most human trials until now [9], seems to be not very promising.

Retinoids are derivatives from vitamin A and are involved in a number of biological processes, e.g., vision, embryogenesis, cell differentiation of blood cells, as well as of skin and tumor cells [10]. Therefore, retinoids are clinically used in oncology, predominantly against acute promyelocytic leukemia [11] and hyperproliferative skin diseases [12]. Moreover, retinoids were found to inhibit inflammation [12], thrombosis [13], platelet adhesion and aggregation [14] and stimulate fibrinolysis [15]. Predominantly all-trans retinoic acid (atRA) and its stereoisomer 9-cis retinoic acid (9cRA) were found to be very potent metabolites of retinol exerting pleiotropic effects on many different biological processes [16]. Previous in vitro studies using rat aortic SMC showed that atRA inhibits platelet-derived growth factor-BB and serum-induced SMC growth. These effects most probably were mediated by five of six retinoid receptors which were expressed by these vascular SMC [17]. In addition, the first in vivo study in rat carotid arteries demonstrated that atRA reduces neointima formation and favors remodeling after endothelial denudation [18].

The current experience with atRA has been achieved in vitro and in vivo with rat aortic smooth muscle cells which do not allow direct comparison to SMC of human origin. Furthermore, the high dose of atRA (30 mg/kg per day) administered into rats might induce severe side-effects in humans. Thus, the aim of our study was to analyze the effects of atRA on human arterial smooth muscle (haSMC) and endothelial cell (haEC)-proliferation and -migration in a transfilter coculture system in which local application of compounds on the endothelium in a broad concentration range can be examined in detail [19,20]. These growth studies were extended by examination of the effects of atRA on phenotypic haSMC-differentiation, on the synthesis of four extracellular matrix constituents, and finally on the degradation of ECM-proteins by matrix metalloproteinases (MMPs). The ECM in the vascular wall includes proteoglycans and fibrous proteins. The latter are classified into two categories: structural (collagen and elastin) and adhesive molecules (laminin, thrombospondin, fibronectin). Thus, examples from each group were examined: collagen-I as an structural protein, thrombospondin-I and fibronectin as adhesive molecules, and biglycan as one of the proteoglycans.

2. Methods

2.1. Cell isolation and culture

Human arterial smooth muscle cells (haSMC) and endothelial cells (haEC) were isolated from specimens of human iliac arteries which were discarded after liver transplantations, as described previously [19]. After mechanical removal of haEC, subcultivation was performed in gelatine-coated (Sigma, Deisenhofen, Germany) plastic flasks using the modified EGM-2 BulletKit. It consists of the basal medium CCMD-130 and the following supplements: hydrocortisone (0.04%), fetal bovine serum (2.0%), vascular endothelial growth factor (VEGF, 0.1%), human recombinant epidermal growth factor (hEGF, 0.1%), long recombinant 3-insulin-like growth factor-1 (R3-IGF-1, 0.1%), hFGF-B (w/heparin, 0.4%), but without heparin, ascorbinic acid and antimycotics (CellSystems, Freiburg, Germany). haSMC were obtained using the explant technique. Subcultivation of haSMC was performed in plastic culture dishes with Waymouth's MB 752/1 and nutrient mixture Ham's F12 (1:1), supplemented with 10% fetal calf serum (FCS, PAA Laboratories, Cölbe, Germany), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL).

Immunocytochemical stainings with specific antibodies against α-smooth muscle actin (Progen, Heidelberg, Germany) and the von Willebrand factor (Boehringer Mannheim, Germany) were used to prove smooth muscle cell and endothelial cell origin, respectively. Microscopic observations showed the characteristic ‘hill and valley’ growth pattern and the ‘cobblestone’ growth pattern, respectively. To avoid immune reactions, both cell types were isolated from the same vessel specimen of the same donor and used in the first three passages. Routine stainings with the DNA dye DAPI (4,6-diamidino-2-phenylindole-dihydrochloride; Boehringer Mannheim) were used to exclude mycoplasma contaminations [4].

2.2. Test compound

All-trans retinoic acid was purchased from Sigma (Deisenhofen, Germany), dissolved in pure ethanol to a stock solution of 10 mM, and sterile-filtered. All preparations were performed under light protection and prepared freshly prior to each application. In order to obtain different test concentrations between 0.1 mM and 0.1 nM, serial dilutions of this stock solution were prepared with culture medium. The vehicle ethanol was also analyzed separately for antiproliferative or cytotoxic effects in equivalent concentrations.

2.3. Test assays

2.3.1. Cell proliferation assays

haSMC or haEC subcultures were rinsed with PBS,
trypsinized (Gibco BRL), counted with a Coulter counter (CASY®, I, Schärfe Systems, Reutlingen, Germany), and seeded onto six-well plates at a density of 5 \times 10^4 cells/cm^2. The next day, number of intact cells and the mitotic index were determined and defined as `baseline values at day 1'. Then, increasing atRA-dilutions were added: (a) over the entire cultivation period which was called `non-stop application' meaning that fresh preparations of atRA-dilutions were supplemented after the replenishment of culture media at days 1, 3, 5, and 7; or (b) as a single dose application for 24 h followed by a washout of the drug and further standard cultivation for 6 days without addition of atRA. At day 7, BrDU-ELISA (Colorimetric Cell Proliferation ELISA, Boehringer Mannheim) were performed 18 h after labeling of cells with the thymidine analogue 5-bromo-2'-deoxyuridine (BrDu), and modified MTT-tests after addition of SDS for 6 h and further incubation with the tetrazolium dye MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide, Sigma) for 18 h, as described previously [4]. Final cell numbers were also determined 24 h after the last medium change and addition of atRA using a cell counter. Each concentration was tested in a total of six wells and repeated twice.

2.3.2. Transfilter coculture system

Cocultures using the transfilter system were prepared according to techniques previously described [21]. Nuclepore® (PC MB 50 mm, pore size: 5 μm) polycarbonate filters were purchased from Costar Scientific (Bodenheim, Germany, No. 111213) and coated with collagen I (from rat tail; Sigma). Coated filters (growth area of 8 cm^2) were inserted between an inner and an outer polycarbonate frame. By this construction, two separate compartments were created in which different cell types could be cultured. haEC (2.5 \times 10^4 cells/cm^2) were seeded on the lower filter side. After 24 h, haSMC (2.5 \times 10^4 cells/cm^2) were added to the opposite filter side (upper filter side). The pore size of 5 μm allows active migration of haSMC from the upper to the lower compartment, as shown previously [19,20]. Then, increasing atRA doses were added to the lower compartment (endothelial side) non-stop or for 24 h in order to imitate local drug application at the ‘luminal side’ of an arterial vessel [4,20]. Culture media were then replaced by normal, drug-free medium, which was renewed every 3rd day. After 14 days, cell numbers on each filter side were determined separately after 14-day cocultivation by cell counting after disaggregation with trypsin/EDTA, and compared to controls which were treated with equivalent concentrations of the vehicle ethanol [4]. Each concentration was tested in a total of three cocultures.

2.4. Immunocytochemical stainings

haSMC were seeded on sterile coverslips and atRA-treated as described above. After 7 days, cells were fixed with cold methanol and labeled with monoclonal anti-heavy chain myosin (Sigma, 1.0 μg/ml) and monoclonal anti-smooth muscle α-actin (Progen, 2.0 μg/ml) antibodies. Secondary labeling was achieved with goat anti-mouse IgG, FITC-conjugated (Sigma, 40.0 μg/ml) or sheep anti-rabbit IgG, Cy3-conjugated (Sigma, 10.0 μg/ml). For additional nucleus staining, haSMC were rinsed and incubated for 30 min with DAPI (250 ng/ml in methanol, Boehringer Mannheim) prior to the incubation with primary antibodies.

2.5. Northern blot analysis

haSMC were cultured in 75-cm^2 plastic flasks until reaching subconfluency (cell density 20,000/cm^2). Then, cultures were treated with increasing atRA concentrations. After 48 h, total RNA was isolated and agarose/formaldehyde gels were loaded with 10 μg total RNA per lane which was resolved by electrophoresis. Blotting, hybridization and detection techniques with digoxigenin-labeled probes were performed according to our previously described methods [22,23]. Hybridization solutions (10 ng cDNA/10 ml) of the following cDNA combinations were used (resulting band in parenthesis): human thrombospondin-1 (TSP-1, 7.0 kb; a gift from Dr Jack Lawler, Boston) and human TGF-β1 cDNA (2.4 kb; ATCC, Rockville, MA, USA); human procollagen type 1 (7.2 and 5.5 kb, clone Hf 677; ATCC) and human biglycan cDNA (2.8 kb; a gift from Dr Larry Fisher, Bethesda); human fibronectin (7.8 kb; ATCC) and human α-smooth muscle actin cDNA (1.8 kb; ATCC). Simultaneously, membranes were hybridized with the cDNA of the housekeeping gene GAPDH (1.4 kb; Clontech, Palo Alto, CA, USA).

2.6. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

haSMC were seeded into 75-cm^2 culture flasks at a density of 20,000 cells/cm^2. Cells were allowed to attach and spread overnight. Then, cultures were serum depleted for 24 h because fetal calf serum per se possesses gelatinolytic activity. Then, increasing doses of atRA (0.01–100 μM) were added. After 6 h haSMC were stimulated with phorbol myristate acetate (PMA, 100 ng/ml, Sigma) or tumor necrosis factor (TNF-α, 10 ng/ml; R&D Systems). Supernatants were collected after 12, 24, 48 and 72 h and concentrated with Centrisart-C 30 columns (Sartorius). Samples were normalized for total protein using the Bradford assay with bovine serum albumin (BSA) as standard.

2.6.1. Western blot analysis

Samples of 15 μg/lane and 10 ng marker/lane (Prestained Protein Molecular Weight Marker, Gibco BRL Life Technologies) were subjected to 15% SDS–PAGE in a
modification of the method described by Laemmli (1970) [24] with the addition of mercaptoethanol (reducing conditions) [25]. After transfer to a nitrocellulose membrane (Hybond C™, Amersham) and blocking, membranes were incubated for 1.5 h with the monoclonal anti-human MMP-2 or MMP-9 antibodies (both from R&D Systems) and the control protein MMP-9 (Calbiochem). Detection was performed using a secondary horseradish peroxidase-linked anti-rabbit antibody (1:500 in blocking buffer) and the ECL™ chemiluminescence system (Amersham).

2.6.2. Zymography
Samples (40 μl/lane) underwent SDS–PAGE at 4°C in 7.5% polyacrylamide gels containing 2 mg/ml gelatine (Sigma). SDS was then removed by rinsing twice with 2.5% Triton X-100 (Sigma). For enzyme activation, gels were incubated overnight at 37°C with incubation buffer containing 50 mM Tris, 10 mM CaCl₂, 0.05% Brij, pH 7.3. Gels were then stained with Coomassie brilliant blue R-250 (Sigma).

2.7. Densitometric evaluation
The intensity of the hybridization bands (Northern blots) and protein bands (Western blots, zymography) was quantitated by densitometry on the X-ray film using an optical color scanner (Agfa). Specific cDNA bands were normalized for loaded RNA by relating each band to the corresponding GAPDH cDNA signal using the MDS ImageMaster Software (Gibco/Kodak).

2.8. Statistics
All values are expressed as mean±S.E.M., whether in numbers or charted. Differences between groups were assessed by unpaired t-test (2 groups) or one-way ANOVA (>2 groups). Subsequent multiple comparisons for ≥3 groups were performed only if one-way ANOVA reached statistical significance (P<0.05) using the Student-Newman-Keul’s test to compare all pairs or Dunnett’s post test to compare each group against control. All data satisfied the requirements of normally distributed samples of equal number between groups. Differences between means were considered statistically significant if P<0.05 and highly significant if P<0.01.

3. Results
3.1. Effects of atRA on haSMC monocultures
atRA caused a dose dependent inhibition of haSMC-proliferation after 7-day incubation time versus controls treated with equivalent doses of the solvent ethanol (Fig. 1). The vehicle ethanol, used in concentrations equivalent to 0.01 nM–10.0 μM atRA, exerted no significant growth inhibitory effects on haSMC and haEC-growth. Cell

![Fig. 1. atRA-induced inhibition of haSMC- and haEC-growth. Cells numbers, mitotic indices and mitochondrial activities at the commencement of treatment (day 1) are shown as single graph points below the 100% values of controls (day 7) and labeled as ‘baseline values, day 1’. (a) Comparison of percentage of cell numbers of haEC and haSMC after 7 days non-stop incubation with atRA measured by cell counting assays. Significant growth inhibitory effects on haSMC (P<0.01 vs. controls) were found in a concentration range between 0.1 and 100.0 μM atRA. In contrast, haEC-growth was stimulated first by lower atRA doses but was also inhibited at higher doses between 1.0 and 100.0 μM atRA. In contrast, haEC-inhibition curve runs in parallel with the inhibition curve of haSMC. However, the haEC curve was completely shifted to higher atRA-concentrations. (b) Comparison of the mitotic activity of haSMC after single dose (24 h) and non-stop application (7 days) of atRA determined by BrdU-ELISA. (c) Comparison of the mitochondrial activity of haSMC after single dose (24 h) and non-stop application (7 days) of atRA determined by MTT tests. Data are shown as mean±S.E.M. values.
counting assay performed at haSMC (Fig. 1a) resulted in an IC_{50} of 0.022 μM and an IC_{max} of 10.0 μM. At this specific maximum inhibitory concentration of 10.0 μM, a more than 60% growth inhibition was observed (38.02±2.76%, \( P<0.01 \)) compared to controls (100.0±4.36%). This value measured at day 7 demonstrated a nearly complete growth inhibition when compared to the ‘baseline value at day 1’. The dramatic decrease of the curves at the highest dose of 100.0 μM (4.06±3.95%) below the ‘baseline values at day 1’ indicated that additional unspecific cytotoxic or apoptotic effects overlapped the specific inhibition.

To determine mitotic index of haSMC after 7 days, BrdU-ELISA were performed comparing non-stop with single dose (24 h) application of increasing atRA-concentrations (Fig. 1b). After non-stop application, the IC_{50}, determined at 0.05 μM, was found in a comparable concentration range as described for the cell counting assay. Shortening of the application time (24 h), however, resulted in a shift of the inhibition curves towards higher concentrations (Fig. 1b) which was reflected by an increased IC_{50} of 20.0 μM.

As also shown in Fig. 1a, the inhibition curve of haEC runs in parallel to the haSMC-curve but the total curve was shifted toward higher atRA-doses. The percentage difference of two consecutive values on the haEC-curve compared to the haSMC-curve was nearly identical. However, the absolute inhibitory effect of each atRA-concentration on haEC-growth was less potent than on haSMC-growth (a difference of ~20–30%) which was also demonstrated by the increased IC_{50} of 97.0 μM which was more than three orders of magnitude higher than that found for haSMC. The maximum specific inhibitory effects were measured at 100.0 μmol. In contrast to the very strong specific inhibition of haSMC (62%) at 10.0 μM, the inhibition of haEC at this dose was just 28% (72.02±5.77 vs. 100.0±1.91%) and at the highest dose of 100.0 μM only 50% (49.76±3.95%). Even at 100.0 μM, the values did not fall below those measured as baseline values at day 1. Moreover, a slight stimulatory effect was induced by 0.1–1.0 nM atRA. MTT-tests (Fig. 1c) and microscopic observations (data not shown) demonstrated that exclusively the highest test concentration of 100.0 μM exerted additional cytotoxic effects on haSMC resulting in some cell rounding and detachment. Comparable to the cell counting assays and the BrdU-ELISA, MTT-tests demonstrated that the values at 100.0 μM were significantly below the baseline values at day 1 indicating some cell loss. The IC_{50} for the mitochondrial activity of haSMC was determined at 0.5 μM after non-stop incubation and at 85.0 μM after single dose application for 24 h.

### 3.2. Effects of atRA on transfilter cocultures

As previously shown, activated haEC in the log-phase of cell growth influence haSMC-proliferation and -migration in transfilter cocultures in a stimulatory fashion by the secretion of growth factors [19,21,22]. Based on these findings, effects of atRA on haSMC-proliferation and -migration in coculture with proliferating haEC were evaluated (Fig. 2). haSMC of controls reached confluence 5–7 days after seeding. Non-stop application of increasing atRA-doses into the lower (haEC)-compartment for 14 days reduced cell numbers on both filter sides dose dependently. Significant effects for haSMC-proliferation were found at doses ≥0.1 μM and for haSMC-migration even at much lower doses ≥0.001 μM (\( P<0.01 \) versus controls). The IC_{50} for haSMC-proliferation and -migration was determined at micromolar to nanomolar atRA-doses.

**Fig. 2.** Growth inhibition of haSMC in transfilter cocultures with proliferating haEC. (a) Effect on haSMC-proliferation and -migration after a single dose application of atRA for 24 h applied to the endothelial side. Cell numbers were determined 14 days after drug replacement by standard medium. (b) Effect on haSMC-proliferation and -migration after non-stop application of atRA for 14 days applied to the endothelial compartment. Solid bars show haSMC numbers on the upper filter side, broken bars the total of haEC and migrated haSMC on the lower side. Data are shown as mean±S.E.M. (*\( P<0.05 \), **\( P<0.01 \) vs. controls).
(9.0 and 0.02 μM, respectively). In contrast, single dose application of atRA stimulated haEC-growth on the endothelial side first at low doses ≥ 0.1 μM whereas haSMC-migration was inhibited just slightly. After application of concentrations ≥ 1.0 μM, both haSMC-proliferation and migration were inhibited significantly. In comparison to the IC₅₀ after non-stop incubation, the IC₅₀ for haSMC-migration after single dose application was shifted toward higher micromolar atRA-doses (1.6 μM). Cell numbers, however, did not fall below those seeded at day 1 indicating that atRA exerted no significant cytotoxic effects on cocultures. These results were confirmed by immunohistologic examinations after double stainings with specific antibodies against the von Willebrand factor and α-SM-actin. Control cultures showed a multilayer (five to six layers) of haSMC on the lower filter side which was covered by a single confluent endothelial cell lining. After treatment with 1.0 μM atRA, the multilayer was reduced significantly on two to three layers whereas the endothelial cell lining was still intact.

3.3. Effects of atRA on cytoskeletal filament formation

Immunofluorescence stainings of haSMC treated with increasing atRA-doses for 48 h demonstrated a striking increase in both α-smooth muscle actin and heavy chain myosin formation. The total number of cells stained positively was increased compared to controls, but also the density of intracellular filaments within each single cell was augmented (Fig. 3). Accordingly, Northern blot analysis after 48 h demonstrated an increase of α-smooth muscle actin mRNA-expression, predominantly at lower nanomolar atRA-doses (between 0.0001 and 0.01 μM) whereas at higher micromolar doses the stimulatory effects were less pronounced (Fig. 4d). This phenomenon might be due to some unspecific cytotoxicity overlapping the specific effects slightly. At 100.0 μM, it was not possible to isolate sufficient amounts of total RNA.

3.4. Effects of atRA on mRNA expression of ECM proteins

Incubation of subconfluent haSMC with atRA in increasing concentrations for 48 h resulted in a dose-dependent inhibition of thrombospondin-1 (TSP-1) mRNA-expression with significant effects at atRA doses ≥ 0.1 μM (Fig. 4a) determined by densitometric evaluation. These results correspond with the dose-dependent inhibition of
Fig. 4. Effect of atRA on mRNA-expression of several ECM-proteins analyzed at subconfluent haSMC. Representative Northern blots of two independent experiments after 48 h. (a) Dose dependent inhibition of thrombospondin-1 (TSP-1) mRNA-expression with significant effects at atRA doses ≥0.1 μM determined by densitometric evaluation. TGF-β1 mRNA-expression was not influenced significantly by atRA. (b) Collagen-1 mRNA expression was slightly increased after treatment with 0.001–0.1 μM atRA. At the highest dose of 100.0 μM, inhibitory effects were caused by overlapping toxic effects. The small proteoglycan biglycan was not influenced by atRA. (c) Fibronectin mRNA expression was slightly inhibited at atRA doses ≥0.1 μM determined by densitometric evaluation. (d) Stimulation of α-smooth muscle actin expression, predominantly at nM atRA-doses. The weaker signal at 0.1 μM was due to loading problems shown by the corresponding GAPDH signal.

atRA on haSMC-proliferation, as shown in Fig. 1. The mRNA-expression of the second glycoprotein fibronectin was also inhibited, but the effect was less pronounced as shown for thrombospondin-1 (Fig. 4c). Furthermore, TGF-β1 and biglycan mRNA-expression were not influenced by atRA (Fig. 4a,b). Collagen-1 was inhibited exclusively at very high atRA-doses between 10.0 and 100.0 μM (Fig. 4b), which might be also the result of overlapping toxic effects reducing cell numbers, as shown in Fig. 1. In contrast to thrombospondin-1 and fibronectin, a slight stimulatory effect on collagen-1 mRNA expression was found in lower doses between 0.01 and 0.1 μM.

3.5. Effects of atRA on MMP-2 and MMP-9 synthesis

SDS–PAGE zymography analysis was performed on haSMC monocultures in order to study synthesis of the metalloproteinases MMP-2 and MMP-9 which can be identified by their gelatinolytic activity. Furthermore, protein synthesis was analyzed by Western blotting. The slight differences in the level of the bands (they are not all in a straight line across the gel) were caused by technical problems with the running of the gel. Supernatants were examined after 12-, 24-, 48- and 72-h cultivation time. Unstimulated haSMC constitutively secreted MMP-2 but not MMP-9. Significant gelatinolytic activity (Fig. 5a, lower lanes) and protein expression (Fig. 5a, upper lanes) of MMP-9 was found after ≥12-h stimulation with phorbol ester (PMA). In contrast to PMA, TNF-α did not induce gelatinolytic activity (Fig. 5b) and protein synthesis (Fig. 5c) of MMP-9 in haSMC significantly after 24- or 72-h incubation.

Preincubation of haSMC with atRA 6 h prior to the stimulation with PMA resulted in a dose dependent inhibition of the gelatinolytic activity, as well as protein expression of MMP-9 in a concentration range between 0.01 and 100.0 μmol/l which was found first 12 h after stimulation with PMA (Fig. 6a) and which persisted for up to 72 h (Fig. 6b–d). Western blotting demonstrated also an partial inhibition of MMP-2 synthesis at highest atRA-
doses between 10.0 and 100.0 μM, predominantly after 24, 48 and 72 h (Fig. 6b–d), whereas zymography showed a remarkable inhibition of enzymatic activity already at lower atRA-doses ≥1.0 μM.

4. Discussion

This in vitro study provides for the first time combined data about the effects of all-trans retinoic acid on human arterial smooth muscle cell proliferation, migration, differentiation, ECM-synthesis and ECM-degradation. The advantage of the used monocultures and cocultures is that primary cultures isolated from human arteries were used in very early passages to preserve most of the in vivo properties of normal arterial cells in humans [19,20]. The transfilter coculture model was developed to imitate the morphology of the arterial vessel wall allowing cell-to-cell interactions by the secretion of PDGF-AB, TGF-β1 and other mediators [21,22].

First, the results with monocultures showed a dose dependent inhibition of haSMC-proliferation after non-stop incubation with 0.001–10.0 μM atRA. This inhibition was uniformly found in all three independent assays used. In addition, analysis of mitochondrial activities indicated that the highest dose of 100.0 μM atRA exerted unspecific toxic or apoptotic effects on haSMC which overlap the specific growth inhibitory effects. The IC₅₀ after 7 days were two orders of magnitude lower than described for animal SMC. In rat SMC, a 50% inhibition was found at 2.0 μM atRA after serum depletion and restimulation with PDGF-BB [17], endothelin [27] or 10% serum [28]. The difference in IC₅₀ indicates that human SMC react more sensitive than rat cells [26] when identical growth assays were used. The effect of atRA was less pronounced in haEC when compared to haSMC, and at lower atRA-doses between 0.0001 and 0.001 μM even a slight growth stimulatory effect could be observed. This is of special importance for clinical application of atRA to prevent restenosis since selective inhibition of haSMC-proliferation without influencing endothelial regrowth is advantageous [29]. In addition, after single dose application of atRA the inhibition curve was shifted towards higher atRA-doses. Thus, for a single local application of atRA higher doses have to be considered to achieve comparable effects as after non-stop application.

When atRA was applied non-stop to the endothelial side of transfilter cocultures for 14 days, a dose dependent inhibition of haSMC-proliferation on the opposite filter side was found. An explanation for the higher IC₅₀ compared to monocultures might be that to reach the target cell, namely the haSMC-multilayer, atRA must cross the endothelial cell lining and the filter pores. Obviously, only part of the applied atRA dosage could indeed reach target cells. Furthermore, haSMC-migration from the upper to the lower filter side towards the endothelial cell lining can be
Fig. 5. Gelatinolytic activity and protein expression of MMP-2 and MMP-9. (a) Supernatants of haSMC-cultures were examined after 12-, 24-, 48- and 72-h cultivation time determined by SDS–PAGE zymography (lower lanes) and Western blotting (upper lanes). Unstimulated haSMC constitutively secreted MMP-2 but not MMP-9. Significant gelatinolytic activity and protein expression of MMP-9 was induced after 12-h stimulation with phorbol ester (PMA). In contrast to PMA, TNF-α did not stimulate (b) gelatinolytic activity or (c) protein expression of MMP-9 in haSMC significantly after 24- or 72-h incubation time.

analyzed additionally and resembles migration of haSMC from the media to the intima in vivo. The total of cell numbers counted on the endothelial side resulted from haEC seeded at day 1 and haSMC which were migrated from the opposite filter side after paracrine stimulation by the secretion of growth factors from haEC. About 3 days after seeding, haEC formed a confluent monolayer and stopped growing. Following this stage, the increase of cell numbers during the next 11 days was caused exclusively by migration of haSMC from the opposite filter side. Thus, the determination of final cell numbers after 14 days mainly represented the result of haSMC-migration. The adequacy of this procedure was confirmed by immunohistochemical examinations indeed showing five to six layers of haSMC on the lower filter side which were covered by a confluent endothelial cell lining, as also described previously [19–21]. After treatment with 1.0 μM atRA, the multilayer was reduced significantly on two to three layers whereas the endothelial cell lining was still intact. After both non-stop incubation and single dose application, haSMC-migration was inhibited by much lower atRA doses than those needed for the inhibition of haSMC-proliferation. However, as described for monocultures, the IC₅₀ was two to three orders of magnitude higher after single dose application. It is of interest that also in the transfilter coculture model haEC-growth was stimulated by nanomolar atRA-doses (0.001–0.01 μM). Since proliferating haEC stimulate haSMC-migration [19–21], low atRA-doses ≤0.1 μM seem to be not potent enough to inhibit haSMC-migration significantly. If higher doses were used or if the drug was applied non-stop, the drug accumulated or the effects were additive. Interestingly, we found in previous studies that the vehicle ethanol in concentrations according to those used to prepare atRA-dilutions (0.001–10.0 μM which corresponds to 0.4% (v/v) ethanol) stimulated haEC-proliferation [4]. Thus, the weaker inhibitory effects of nanomolar atRA doses after single dose-application might be partially counterbalanced by stimulatory effects of ethanol. Taken together, the coculture results again underline the importance of the use of a more complex coculture model as a prescreening experimental setup before moving to in vivo studies.

In contrast to our results, Neuville et al. (1999) found increased migration of rat aortic smooth muscle cells after administration of atRA, whereas matrix metalloproteinases
migration in the intima or plaque would be expected. Whereas an increased matrix deposition may be positive for the stabilization of advanced fibrous plaques avoiding probably acute plaque rupture, the increase of extracellular matrix in restenosis would be negative because it contributes to intimal thickening. However, Northern blot analysis showed additionally that specific extracellular matrix proteins were either inhibited or stimulated by the addition of atRA. Collagen-1 expression was slightly upregulated by the treatment with nanomolar doses of atRA whereas thrombospondin-1 and fibronectin mRNA expression were inhibited dose dependently. However, the inhibition of fibronectin was not as strong as found for thrombospondin-1 which corresponds to the results of Riessen et al. (1999) showing that statins inhibited thrombospondin-1 but not fibronectin mRNA [23]. Thus, fibronectin mRNA expression seems to be more stable than thrombospondin-1 mRNA expression, even after treatment with multipotent drugs. The expression of the small chondroitin/dermatan sulfate proteoglycan biglycan was not altered by atRA. The inhibition of the glycoprotein thrombospondin-1 is of special interest because it was found to act as a mitogen for smooth muscle cells and also stimulate cell migration [32]. Furthermore, it is an activator of TGF-β1, a growth factor which inhibits haSMC growth, as we demonstrated previously [22]. Thrombospondin-1 influences plasmin formation by a complex formation with plasminogen which contributes — in a synergistic manner with MMPs — to ECM degradation and smooth muscle cell migration. In addition, thrombospondin-1 was found to inhibit MMP activity by preventing activation of the MMP-2 and MMP-9 zymogens. Finally, it inhibits endothelial cell migration and angiogenesis and promotes endothelial cell apoptosis [33]. Thrombospondin-1 acts as a kind of immediate-early gene which is expressed immediately after vessel injury [32], whereas fibronectin upregulation is both an early and long-lasting process after arterial injury. Fibronectin influences, through interaction with integrins, motility, differentiation, and many of the cellular responses associated with wound healing and vessel diseases [34]. Relevant amounts of collagen-1 and the small proteoglycan biglycan are expressed much later [35]. Abundant collagen-1 formation is characteristic for advanced atherosclerotic lesions and contributes to the stabilization of the fibrous cap [23]. In accordance with this finding, Hayashi et al. (1995) described that atRA also causes an increase of elastin synthesis [36]. Since fibrous proteins of the ECM can be classified within two categories, structural (collagen, elastin) and adhesive molecules (laminin, thrombospondin, fibronectin), our results indicate that structural proteins seem to be stimulated by atRA whereas adhesive molecules are inhibited. Finally, the expression of the growth factor TGF-β1 which was found to stimulate the synthesis of a variety of ECM-proteins [31] and inhibit SMC-proliferation [22] was not changed by atRA. Thus, stimulation of collagen-1 expression by atRA is not the result of...
an primary effect on TGF-β1 expression. These results show that atRA exerts pleiotropic effects on vascular cells and that the total amount of matrix and the net effect of atRA depends on a variety of different factors.

In addition to these effects, haSMC-differentiation was influenced by atRA. Previously, we found, in accordance with Patel et al. (2000), that under normal culture conditions α-smooth muscle actin mRNA and protein synthesis are suppressed in actively proliferating SMC isolated from iliac arteries and increase again as the cultures become confluent and cell replication ceases [37]. Thus, α-smooth muscle actin expression depends on the growth rate and cell density, as also described by others [38]. The present study showed that atRA caused an increase in smooth muscle α-actin and heavy chain myosin protein content in actively proliferating cultures. Furthermore, nanomolar atRA-doses stimulated α-smooth muscle actin mRNA-expression whereas the increase at higher micromolar doses was reduced by overlapping cytotoxic effects reducing cell numbers slightly, as also described for collagen-1 mRNA expression. We conclude that atRA might be beneficial for the stabilization of the fibrous cap by the stimulation of haEC and haSMC differentiation toward a non-activated, fully differentiated phenotype which is commonly associated with vascular cells residing in the normal media and in the fully differentiated fibrous cap of advanced lesions [39]. Non-activated, highly differentiated cells are not capable of synthesizing matrix degrading proteins. Thus, they cannot contribute to the weakening of the plaque. As a consequence, the stabilization of the fibrous cap increases which is supported by the direct inhibition of MMP-2 and MMP-9 synthesis in haSMC and by the increase in collagen-1 synthesis after atRA-treatment.

An important question is if the described effective in vitro concentrations of atRA can be achieved in vivo. Unfortunately, both the in vivo and in vitro half-lives determined for atRA are relatively short. Miundi et al. (1992) reported in APL-patients that after a single oral dose of 45 mg/m² atRA, the C_{pmax} in plasma was reached after 1–2 h and decreased to <10 ng/ml by 8 h. The peak plasma concentration of atRA was 346±266 ng/ml (=1.15±0.88 μM) and the half-life was 0.8±0.1 h [11]. Lefebvre et al. (1991) found 24 h after a single oral dose of 45 mg/m² a peak concentration of 0.03–2.5 μg/ml (=0.099–8.31 μM) [40]. When compared to the present in vitro-study with human arterial cells as well as to the study of Lansink et al. (1997) with HUVECs [41], 0.1–10.0 μM atRA are required to induce optimal effects on vascular cell growth and t-PA synthesis, respectively. The IC_{50}s determined for haSMC (monocultures: 0.0005–20.0 μM; cocultures: 0.02–9.0 μM) correspond closely with ordinary therapeutic doses. HPLC analysis of human endothelial cells after incubation with 1.0 μM atRA showed that the disappearance of atRA was biphasic: an initial phase of 8 h with a half-life of 8 h and a second phase during the next 16 h with a half-life of 2 h which might reflect induction of atRA-metabolizing capacity or saturation kinetics because incubation with 20 nM atRA showed a monophasic disappearance with a half-life of 1.4 h [40]. However, despite the rapid elimination of atRA, there is striking in vitro and clinical activity after treatment of APL-patients with atRA indicating that also lower rest doses in the nanomolar range present after metabolization might be sufficient to inhibit vascular cell growth or that active metabolites are formed which contribute to the effects of the parent drug atRA. Finally, for the prevention of restenosis atRA might be applied locally by the use of porous balloons or stents which allows the use of higher doses as after oral application without side-effects.

There are only sparse data available regarding the underlying mechanisms by which atRA elicits its effects on vascular cells. Previously, Miano et al. (1996) showed that five of the six nuclear retinoid receptors (RAR-α, -β, -γ and RXR-α, -β) were expressed in rat aortic SMC and that atRA might bind and activate the expression of at least the RAR-β receptor [17]. We were able to detect the five nuclear receptors RAR-α, -β, -γ and RXR-α, -β on haSMC by RT-PCR which were expressed to different extents (unpublished data), but further work is needed to elucidate the exact mechanisms after binding of retinoids to these receptors.

In conclusion, we have demonstrated that atRA exerts pleiotropic effects on a variety of processes involved in the formation of advanced atherosclerotic lesions as well as in restenosis, whereas most other compounds studied previously were only capable of inhibiting one step, e.g. accelerated smooth muscle cell growth. Further in vitro and in vivo studies are required to prove the toxicity and efficacy of atRA after clinical applications against arterial vessel diseases to elicit the systemic and local cellular mechanism of the classical vitamin-A derivative atRA and to develop potentially more receptor-specific, non-toxic synthetic derivatives.

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