Effects of local all-trans-retinoic acid delivery on experimental atherosclerosis in the rabbit carotid artery

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Abstract

Background: Retinoids regulate a variety of biological processes and play an important role in cell differentiation and proliferation. All-trans retinoic acid (atRA) is known to inhibit smooth muscle cell growth and thus is supposed to have favorable effects on the incidence of restenosis after percutaneous coronary interventions. The broad biological spectrum, however, leads to numerous severe side effects which limit the clinical use of a systemic application of atRA. In order to avoid systemic side effects, local delivery of atRA is preferable. The aim of this study was to evaluate the effects of atRA on the response to injury in a second-injury model of experimental balloon angioplasty.

Methods: After induction of a fibromuscular plaque in the right carotid artery of 40 New Zealand rabbits, 35 animals underwent balloon angioplasty of the preformed plaque formation. Subsequent local atRA delivery (10 ml, 10 μM) with the double-balloon catheter was performed in 15 animals. Five animals received vehicle only as sham controls, and five animals were solely electrostimulated; 15 animals served as control group with balloon angioplasty only. Vessels were excised 7 days (n = 15) and 28 days (n = 30) after intervention. Immunocytochemistry with antibodies against smooth muscle α-actin and myosin, bromodeoxyuridine, macrophages, collagen I and III and von Willebrand factor was performed. Quantitative analysis was done by computerized morphometry.

Results: After local atRA delivery in vivo, the extent of stenosis was markedly reduced with 21.7 ± 8.3% (mean ± S.D.) 4 weeks after intervention compared to 31.8 ± 13.4% in balloon-dilated animals (P = 0.0937). Both a reduced early neointimal proliferation (P = 0.0002) and an increase in overall vessel diameter (4 weeks after intervention, P = 0.0264) contributed to a limitation of restenosis in atRA-treated animals. Immunocytochemistry revealed a more intense α-actin staining pattern after local atRA therapy indicating redifferentiating effects of atRA on vascular smooth muscle cells. Conclusions: Local delivery of atRA led to limitation of restenosis formation in this animal model. The concept of a local atRA therapy might be a promising way to exploit the potential of atRA for vascular indications while minimizing the severe side effects of systemic retinoid therapy.

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1. Introduction

Retinoids, the natural and synthetic derivatives of vitamin A, are known to regulate a variety of important biological processes. Their importance for vision, skin, embryonic growth and reproductive processes is well known. Mediated by nuclear receptors, retinoids are key elements in the regulation of cellular proliferation and differentiation directly on a genetic basis [1–5]. Retinoids preserve the homeostasis of cell differentiation and prolif-
eration and are successfully used as therapeutic agents for many proliferative diseases. They are widely used in skin diseases that are caused by a dysregulation of cellular proliferation like psoriasis or ichthyosis. Furthermore, retinoids are used in the prevention and therapy of different cancers and for treatment of acute promyelocytic leukemia [6–8]. Because of their important role as regulators of cellular growth, differentiation, morphogenesis and metabolism, retinoids may also be of use to modify the proliferative response after arterial vessel wall injury.

It has already been shown that retinoids exert growth inhibitory effects on vascular smooth muscle cells (smc) [4,9,10]. In vitro experiments we found that atRA caused a dose-dependent inhibition of human smc-growth, whereas human endothelial cell-growth was inhibited less potently. Additionally, proliferation and migration of human smc were inhibited even after single-dose application in an in vitro transfilter-coculture model [11]. Two recent in vivo studies also indicated modulation of smooth muscle cells in balloon-injured rats and rabbits after systemic therapy with atRA, a biologically active metabolite of vitamin A, and suggested favorable effects in the limitation of restenosis [12,13]. However, the systemic doses of atRA used in these experiments were several times higher than the dosage used for induction therapy in human malignant diseases [14]. Therefore we sought for a way to exploit the positive properties of atRA while concurrently minimizing the severe side effects of a systemic retinoid therapy. This study was performed to evaluate the effects of a local atRA delivery via the double-balloon catheter on experimental atherosclerosis in the rabbit carotid artery.

2. Methods

2.1. All-trans-retinoic-acid

AtRA (Sigma, Deisenhofen, Germany) was prepared under reduced lighting conditions. The substance was dissolved in 100% ethanol and filter-sterilized. The resulting stock solution (2.5 mM) was then diluted with sterile 0.9% NaCl-solution to a concentration of 10⁻⁵ M. For each animal 10 ml of 10⁻⁵ M atRA were prepared freshly under the same conditions as described above. Sham-treated animals received 10 ml of sterile 0.9% NaCl-solution without atRA.

2.2. Animal study

White New Zealand rabbits (2.8–3.4 kg) were obtained from Thomae (Biberach, Germany) and kept in our institution for 2 weeks before inclusion in the study. The study protocol was reviewed by the ethical committee on animal research of our institution and was found to conform with the Guide for the Care and Use of Laboratory Animals. The rabbits were housed individually with 12-h light periods. Animals were weighed and behaviour (active or apathetic, friendly or aggressive, etc.) was monitored daily during the whole study as part of the screening for side effects of the treatment. We looked daily for rapid weight gain as a hint of edema and other clinical signs like dyspnea (pleural effusion), inspected the snout for signs of cheilitis and stools for diarrhea. The ongoing study was closely supervised by the veterinarian of our institution. In order to induce a fibromuscular plaque in the right carotid arteries of the rabbits, the electrostimulation model was used in this study as described previously in detail [15–17]. In brief, two graphite-coated gold electrodes were implanted in the adventitia of the common carotid artery, held in position by a Teflon cuff and then subcutaneously connected to a small plastic socket attached to the skull. Using an external stimulation unit, constant direct current impulses were then applied twice daily for 28 days, 18 000 impulses in the morning and 9000 in the afternoon. Additionally, the animals were fed a 0.5% cholesterol diet (Altromin, Lage, Germany) during the electrostimulation period in order to produce a cholesterol-rich plaque. This combined procedure leads to an eccentric plaque, growing below the anode. Animals were randomly assigned to the atRA-group or the three different control groups before the catheter interventions. A schematic survey of the study protocol is shown in Fig. 1. After the induction of an eccentric plaque formation in 40 New Zealand rabbits by repeated electrical stimulation, balloon angioplasty was performed in 35 animals under...

![Fig. 1. Scheme of the experimental study protocol. (I) Animals that were solely electrostimulated served as pre-interventional control group. (II) Control animals that underwent balloon angioplasty only. (III) Intervention groups after local delivery of 10 ml 10 μmol/l atRA with the double-balloon catheter. (IV) Sham control animals after local delivery of 10 ml 0.9% NaCl-solution (vehicle) with the double-balloon catheter.](https://academic.oup.com/cardiovascres/article-abstract/57/2/544/307884)
2.4. Immunocytochemistry and histomorphometry

In order to identify replicating cells, immunohistological staining was performed with a monoclonal antibody against BrdU (Bio Cell Consulting, Grellingen, Switzerland). Vascular smooth muscle cells were identified and analysed with immunohistological anti-α-actin- and anti-myosin heavy chains 1- and 2-staining (Renner, Dannstadt, Germany and Sigma). Macrophages were detected with RAM 11 antibody [18,19]. In order to investigate the effects of atRA on endothelial cells and re-endothelialization after intervention, cross-sections were stained with a polyclonal antibody against von Willebrand factor (goat anti-human factor VIII-related antigen, Atlantic Antibodies) confirming the endothelial origin of the luminal cell lining. The histological sections were additionally stained for collagen I and III with polyclonal goat anti-human antibodies (both from Dunn, Asbach, Germany). As chromagen we used either 3-amino-9-ethylcarbazole or 3,3'-diaminobenzidine for all the immunohistological stainings. Controls were done by performing the identical staining procedure but omitting the first antibody. In addition to the immunohistology, histological sections were stained with hematoxylin–eosin and elastica–van Gieson’s stain. Each staining was performed in cross-sections of each vessel segment. α-Actin-, myosin-, collagen I- and III-stained histological sections were semiquantitatively analysed using a 0–4 scaling score (0=absence of staining, 1=only localised staining, 2=diffuse but weak staining, 3=diffuse and medium staining, 4=diffuse and heavy staining). The number of proliferating cells or macrophages was determined by counting the number of BrdU-positive cells and RAM 11-positive cells, respectively, and related to the total intimal or medial cell count. Endothelial regeneration was quantified by relating the number of endothelial cells to the length of the endothelial layer. Elastica–van Gieson’s stained sections were quantitatively analyzed by computerized morphometry using standard software (Bioquant, Bilaney Consulting). The perimeters of the luminal border, internal elastic layer and external elastic layer were manually traced, digitalized, and the enclosed areas were directly calculated on a digitalized pad (Summagraphics, Seymour, CT, USA). Thus, the software used the actual perimeters to allow for a calculation of the area of intima, media and residual lumen. The degree of stenosis was determined as % stenosis=intimal area×100/intimal+luminal area. The microscopic examination of the histological cross-sections was performed by two independent observers, blinded to the type of treatment protocol. Before ultimate analysis, 50 histological sections were randomly analysed by these two observers. The interobserver variability was 8% for the cell counting, 10% for the semiquantitative scoring and 2% for the morphometric measurements.

2.5. Statistical evaluation

The Shapiro–Wilks test was used to test for normal distribution of the results. The data for the semiquantitative staining scores are expressed as median, whereas all other results are expressed as mean±S.D. In order to determine the significance of differences between atRA-treated animals and control animals, analysis-of-variance was per-
formed for the main variables (neointimal proliferation, extent of stenosis) including all groups and considering that the data were obtained at different times. When ANOVA confirmed statistical differences for the analysed variables, the Tukey’s studentized range test was performed to further analyse the differences between the different groups. In order to directly compare the effects of therapy at the end of the study 4 weeks after intervention an additional Student’s t-test was performed for the morphometric data, and the P values are given in the text.

3. Results

3.1. Morphological results

Thrombus formation occurred in a total of three animals, two thrombi (one occlusive and one mural thrombus) were found in the control group 4 weeks after balloon angioplasty alone, and one occlusive thrombus was present in the atRA group 4 weeks after intervention. These vessels were excluded from further quantitative analysis. Vessel trauma induced by angioplasty was detectable in nearly every section 7 days after intervention with loss of endothelium, disruptions in the internal elastic layer, circumscript dissections and sometimes medial compression. No difference between the different groups could be detected. Twenty-eight days after intervention, no internal disruptions were detectable; however in three animals vessel dissections (two in the atRA group and one in the BD group) were observed. In some animals the use of the double-balloon catheter led to a minor additional injury in areas of the vessel wall that had been in contact with the two balloons of the ldd-device: an additional loss of endothelium (up to 50% of the whole luminal cell lining) was observed in two sham animals and three animals after atRA treatment 7 days after intervention. Furthermore, serial cutting of one sham-treated control animal (0.9% NaCl) revealed neointimal proliferation of about two cell layers in areas that were directly exposed to the two balloons of the double-balloon. Macrophages were detected mainly in areas that showed signs of injury and were difficult to count because of cluster formation. The number of intimal macrophages was markedly increased 7 days after intervention compared to preinterventional controls, whereas the difference in macrophage content between the different groups differed only little. Medial macrophages were slightly increased 1 week after intervention. One month after intervention the number of intimal and medial macrophages was close to preinterventional levels. The exact numbers are given in Table 1.

3.2. Effects on proliferation, α-actin staining and matrix composition

The results of the α-actin- and myosin-staining score are shown in Fig. 2. Semi-quantitative scaling was possible in all parts of the vessel wall using α-actin- and myosin-staining. Collagen I staining allowed scaling in the intimal layer only, since only weak staining without differences between the groups was observed in the medial area (Fig. 3). No difference in the staining score for collagen I was observed between all groups (Table 1). Immunohistochemistry for collagen III showed intense and diffuse staining in all vessel compartments in each group and at all points of time without differences (data not shown). Cross-sections from atRA-treated animals exhibited more intense α-actin- and myosin-staining than did control sections after BD 4 weeks after intervention (cf. also Figs 2 and 4). The individual results for intimal and medial proliferation as well as the number of endothelial cells are provided in Table 1. After 28 days of electrostimulation the preinterventional proliferation was low in the medial and intimal layer (1.5±0.7%) and (1.0±0.2%), respectively. Seven

Table 1

<table>
<thead>
<tr>
<th>Data/Mean±S.D.</th>
<th>Pre-interv.</th>
<th>BD 7</th>
<th>NaCl 7</th>
<th>AtRA 7</th>
<th>BD 28</th>
<th>AtRA 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lumen area (mm²)</td>
<td>0.725±0.149</td>
<td>1.136±0.262</td>
<td>1.202±0.483</td>
<td>1.188±0.546</td>
<td>0.898±0.287</td>
<td>1.310±0.362</td>
</tr>
<tr>
<td>Intimal area (mm²)</td>
<td>0.267±0.172</td>
<td>0.378±0.167</td>
<td>0.390±0.079</td>
<td>0.225±0.070</td>
<td>0.445±0.259</td>
<td>0.380±0.128</td>
</tr>
<tr>
<td>Stenosis (%)</td>
<td>25±9.1</td>
<td>23.8±6.1</td>
<td>27.0±10.4</td>
<td>18.6±7.7</td>
<td>31±13.4</td>
<td>21.7±8.3</td>
</tr>
<tr>
<td>Media area (mm²)</td>
<td>0.702±0.194</td>
<td>0.664±0.172</td>
<td>0.905±0.315</td>
<td>0.829±0.240</td>
<td>0.842±0.122</td>
<td>1.077±0.232</td>
</tr>
<tr>
<td>Total vessel area (mm²)</td>
<td>1.693±0.415</td>
<td>2.178±0.372</td>
<td>2.496±0.442</td>
<td>2.242±0.434</td>
<td>2.186±0.330</td>
<td>2.768±0.546</td>
</tr>
<tr>
<td>Intimal proliferation (%)</td>
<td>1.5±0.7</td>
<td>15.9±10.4</td>
<td>15.3±5.7</td>
<td>9.5±6.5</td>
<td>4.0±2.2</td>
<td>3.2±2.7</td>
</tr>
<tr>
<td>Medial proliferation (%)</td>
<td>1.0±0.2</td>
<td>4.4±2.0</td>
<td>4.3±0.9</td>
<td>3.4±2.2</td>
<td>3.3±2.0</td>
<td>1.9±1.4</td>
</tr>
<tr>
<td>Intimal macrophages (%)</td>
<td>1.5±0.6</td>
<td>14.8±8.4</td>
<td>18±3.3</td>
<td>14.3±6.5</td>
<td>3.0±1.2</td>
<td>4.4±0.9</td>
</tr>
<tr>
<td>Medial macrophages (%)</td>
<td>1.5±0.8</td>
<td>1.8±0.8</td>
<td>3.7±1.9</td>
<td>1.7±0.9</td>
<td>3.7±1.9</td>
<td>3.7±5.3</td>
</tr>
<tr>
<td>Endothelial cells/mm</td>
<td>53±12</td>
<td>46±7</td>
<td>41±17</td>
<td>39±12</td>
<td>45±26</td>
<td>40±22</td>
</tr>
<tr>
<td>Collagen I score</td>
<td>0.6±0.5</td>
<td>3.5±0.5</td>
<td>3.8±0.4</td>
<td>3.3±0.4</td>
<td>2.3±1.0</td>
<td>2.2±0.6</td>
</tr>
<tr>
<td>Myosin score</td>
<td>2.4±0.5</td>
<td>1.4±0.5</td>
<td>1.4±1.5</td>
<td>1.8±0.4</td>
<td>2.2±0.4</td>
<td>2.6±1.2</td>
</tr>
<tr>
<td>α-Actin score</td>
<td>3.4±0.5</td>
<td>1.8±0.8</td>
<td>1.6±0.5</td>
<td>2.2±0.7</td>
<td>2.4±0.7</td>
<td>3.4±0.5</td>
</tr>
</tbody>
</table>

Pre-interv., preinterventional control group; BD7(28), balloon dilatation only 7 (28) days after intervention; NaCl, sham-treated animals after local delivery of 0.9% saline solution; AtRA, local delivery of all-trans-retinoic acid.

*No statistical difference compared to preinterventional controls; #statistically different from preinterventional controls.
3.3. Effects on vessel shape

The preinterventional baseline value for the extent of stenosis was 25.0±9.1%. One week after intervention the degree of stenosis in balloon-dilated control animals was 23.5±6.1%, 27.0±10.4% in sham control animals, and 18.6±7.7% in atRA-treated rabbits. Four weeks after intervention the difference between balloon-dilated controls and atRA-treated animals was increased to 21.7±8.3% (atRA) versus 31.8±13.4% (BD), $P=0.0937$. The total vessel area, measured as the area within the external elastic layer, was similar in all groups 1 week after intervention (2.178±0.372, 2.242±0.434 and
2.496±0.442 mm², respectively, but markedly larger in atRA-treated animals compared to balloon-dilated control animals 4 weeks after intervention (total vessel area after 4 weeks: atRA group, 2.786±0.546 mm²; BD-control, 2.186±0.330 mm²; P=0.0264). The results and P values of the morphometric data are also shown in Fig. 6, and a detailed list of the results in the different study groups is given in Table 1.

4. Discussion

Local atRA delivery led to a decrease in early neointimal proliferation, a decrease in the degree of stenosis and an overall enlargement in vessel size in this animal model. The positive results of this study are in agreement with the two recent in vivo studies aimed to inhibit restenosis using a systemic application of atRA [12,13]. Furthermore our results support the possibility of a local therapy of atRA, which might be a crucial concept for a future clinical application.

4.1. All-trans-retinoic acid

Retinoids are lipophilic, have pleiotropic effects and act in very small doses. They have become important therapeutic agents in the treatment of hyperproliferative diseases like leukemia or psoriasis. AtRA, a biologically active metabolite of vitamin A, has been shown to inhibit cell proliferation and migration, platelet aggregation, thrombosis and inflammation. Furthermore atRA promotes cellular differentiation and causes pluripotential embryonal cells to express SMC characteristics and to ‘mature’ cultured SMCs into a contractile phenotype [20,21]. Development of primary atherosclerosis and restenosis after percutaneous interventions are closely linked to cellular proliferation, migration and expression of extracellular matrix. Thus, atRA is a promising agent to target coronary artery disease. However, the clinical use of retinoids is limited by severe side effects [6–8,22,23]. Retinoids are such important teratogens that after the end of treatment with some synthetic retinoids contraception should be maintained for a further 2 years, but still major birth defects after isotretinoin treatment are a big problem [24,25]. It is therefore doubtful if a systemic therapy with doses as high as used in the two cited in vivo studies will find its way into the clinical setting for this indication. Based on these considerations the aim of our study was to test the efficiency of a local therapy concept with atRA in order to minimize systemic side effects.

4.2. Local drug delivery

Since local drug delivery is still hampered by several unresolved problems such as low drug transfer into the vessel wall, short action of the drug, or additional vascular trauma, and the interventional mechanisms of transferring drugs into the vasculature are very limited, it is essential to identify potent drugs with optimized pharmacological properties for local drug delivery [26–38]. The lipophilic properties of atRA facilitate a rapid cellular uptake within a short period of time and the double-balloon catheter allowed passive uptake of atRA into the vessel wall during a period of several minutes. In addition, vascular injury by the use of the double-balloon catheter was very limited. This combination was obviously sufficient in this study to result in the inhibition of neointimal proliferation as well as an increase in total vessel area. Recently very promising results were obtained with drug eluting stents. In fact, there are several potential advantages of active stents including a
prolonged release of potentially more drug. Development in stent-coating techniques is continuing, however, there is also still concern that problems like late thrombosis or necrosis around the stent struts, as observed with brachytherapy, will hamper this new technique [39–41].

4.3. Animal model

For the current study, the electrostimulation model offered several advantages, the most important of which is that it is a second-injury model where the endothelium remains uninjured. A morphologically intact endothelium allows for an identification of the second injury caused by balloon dilatation. This is especially important for the technique of local drug delivery, where additional injuries to the vessel wall may occur. Furthermore, the electrostimulation model induces a defined plaque, which is comparable in size in all animals. The combined approach of electrostimulation and cholesterol feeding induces plaques that are morphologically very similar to early human plaques, because characteristic features like proliferation of smooth muscle cell, deposition of cholesterol and invasion of monocytes are present [15–17].

Although the stimulation electrode is only loosely attached, this model, however, allows only for a limited examination of the adventitial layer. Analysis of the perivascular space was therefore generally excluded in this study.

4.4. Results of this study

There are two studies about retinoids for restenosis in literature using systemic atRA delivery in rats and rabbits, therefore our favorable effects of a local atRA delivery.
have to be compared to these results. The increase in total vessel area in this study was also observed in the two systemic atRA studies in rats and rabbits. Miano et al. found depression in peak DNA-synthesis in the medial layer as well as an increase in vessel wall perimeter in the rat carotid artery [12]. Wiegman et al. also described an overall vessel enlargement in the rabbit femoral artery; however, no significant difference in smc-proliferation 72 h after intervention was noted [13]. Although our results were also obtained in rabbits, they correspond—with regard to proliferation—to the results of Miano et al., possibly because these experiments were also performed in carotid arteries. Our results of the α-actin, myosin and collagen staining correspond again to the data of Wiegman et al., who found significant more α-actin staining in treated animals than in controls, whereas collagen staining was similar in the two groups. Although in both of these studies only semiquantitative scoring was used, there is strong evidence in literature of differentiating effects of retinoids in different species, data which also support our observations. We have recently published a paper in which the effects of atRA on human mono and transfiber co-cultures were studied. In vitro, a striking increase in both α-smooth muscle actin and heavy chain myosin formation was found after atRA treatment. Both, the total number of cells stained positively and the density of intracellular filaments within each single cell was augmented. In this study we also looked at RNA expression, and accordingly, Northern blot analysis demonstrated an increase of α-smooth muscle actin mRNA-expression after atRA-treatment [11]. It is therefore reasonable to speculate that some of these results can also be reproduced in human coronary arteries. In these in vitro experiments we also observed a less potent growth inhibition in human endothelial cells after atRA treatment (IC\textsubscript{50} at 97 μM) compared to smooth muscle cells (IC\textsubscript{50} at 0.022 μM). This might explain that in our study we found no statistical difference in the number of endothelial cells between animals that were treated locally with atRA and balloon-dilated controls.

### 4.5. Limitations and significance

A single animal model does not sufficiently represent the pathomechanisms involved in human restenosis. Moreover this in vivo model gives no insight into the molecular mechanisms by which atRA has caused favorable effects on restenosis limitation. Numerous and complex pathways have already been identified for retinoids in vitro (two nuclear receptor families, three subgroups each, multiple isoforms, tissue- and time-specific expression, different cellular binding proteins, etc.). Therefore further studies will be necessary to unveil at least some elements of the mode of action of atRA in vasculature.

The results of this study are presented with a certain reserve as regards statistical power. Animals are individuals and the group sizes in this study are rather small, therefore the results of this animal study rather reflect trends. Another serious limitation in this study is the lack of tissue or blood levels of atRA. It is well known that after ldd the applied drug will be washed out rapidly. However, the more important question is the biological efficacy of this limited and short application [42]. Like paclitaxel that leaves its ‘fingerprints’ in the cell via an altered cytoskeleton when the drug itself is long gone, atRA might induce some cellular changes like promotion of smc differentiation with its subsequent effects on proliferation, migration and matrix production that hold on after the drug itself has vanished. Now that the concept of...
a local atRA therapy has shown first promising results, other local concepts arise, including the incorporation of atRA into a coated stent with a presumably prolonged and higher drug release.

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References


