Differential localisation of the renin–angiotensin system in de-novo lesions and in-stent restenotic lesions in in-vivo human coronary arteries

Lodewijk J. Wagenaar, Ad J. van Boven, Allard C. van der Wal, Giovanni Amoroso, René A. Tio, Chris M. van der Loos, Anton E. Becker, Wiek H. van Gilst

Abstract

Objective: Different components of the renin–angiotensin system (RAS) have been demonstrated in atherosclerotic plaques. However, the involvement of the RAS in in-stent restenosis is not clear. We studied the differential immunolocalisation of angiotensin converting enzyme (ACE) and the angiotensin II type 1 (AT1) receptor in de-novo stenotic lesions and in-stent restenotic lesions in human coronary arteries.

Methods: Using a pullback atherectomy catheter, biopsies from de-novo coronary lesions \( n = 19 \) and in-stent restenotic lesions \( n = 19 \) were obtained. The biopsies were immunostained for vascular smooth muscle cells (VSMCs), macrophages, ACE and the AT1 receptor.

Results: In biopsies from de-novo stenotic lesions ACE-positive macrophages were more numerous than in in-stent restenotic lesions \( P < 0.002 \). Moreover, in the latter lesions, ACE-positive macrophages decreased when the time interval of stent implantation was longer. On the other hand, in-stent restenotic lesions contained predominantly young VSMCs, which abundantly expressed AT1 receptors.

Conclusions: Lesional ACE expression is not a prominent feature of in-stent restenotic lesions. In contrast, AT1 receptors are abundantly expressed on young VSMCs. In de-novo lesions ACE and AT1 receptors were found on macrophages and VSMCs, which were present in all specimens.

Keywords: Renin angiotensin system; Receptors; Coronary disease; Stents; Restenosis

1. Introduction

The involvement of the renin–angiotensin system (RAS) in the occurrence of atherosclerotic plaque instability has already been addressed. An increased accumulation of angiotensin-converting enzyme (ACE), of angiotensin II, and of angiotensin II type 1 (AT1) receptors has been demonstrated in human coronary arteries in the course of acute coronary syndromes, in relationship with the amount of activated macrophages present in the atherosclerotic plaque [1–3]. RAS activity in stable atherosclerotic lesions is still under investigation [4–6].

Despite the diffuse utilisation of stents, restenosis after percutaneous coronary interventions (PCI) still develops in 10–21% after stent placement [7]. Histopathological differences suggest a different pathogenesis for in-stent restenotic and atherosclerotic de-novo lesions.

In de-novo coronary atherosclerosis, the angiotensin converting enzyme (ACE) has emerged as one of the factors involved in the process of lesion formation, since an increased accumulation of ACE in humans has been demonstrated in atherosclerotic coronary and carotid arteries [4–6].

*Corresponding author. Department of Cardiology, Thoraxcenter, University Hospital of Groningen, Groningen, The Netherlands. Tel.: +31-50-363-2811; fax: +31-50-363-2812.
E-mail address: w.h.van.gilst@med.rug.nl (W.H. van Gilst).

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In-stent restenosis seems to be caused exclusively by neointimal proliferation [8]. In-stent restenotic lesions contain mainly smooth muscle cells and show more active proliferation of smooth muscle cells than native lesions and even restenotic lesions after balloon angioplasty [9]. Given the fact that smooth muscle cell proliferation can be induced by angiotensin II via the AT1 receptor under experimental conditions [10–12], we investigated the potential involvement of the renin–angiotensin system in the development of human in-stent restenosis.

For this purpose we used atherectomy specimens retrieved from patients with in-stent restenotic lesions, in which the immunoreactivity of smooth muscle cells and inflammatory cells with anti-ACE and anti-AT1 receptor antibodies were evaluated. Moreover, the results were compared with atherectomies from de-novo atherosclerotic lesions subjected to similar immunohistochemical procedures.

2. Methods

2.1. Study population

Consecutive patients with recurrent stable angina or inducible ischemia after coronary stenting, and the angiographic finding of in-stent restenosis were enrolled (n=19). In-stent restenosis was defined as an in-stent lumen diameter of less than 50% of the vessel. A second population of symptomatic patients with significant de-novo coronary lesions, matched for age, gender and lesion location was also selected (n=19). These patients had stable angina and type B2 or C lesions in the proximal left anterior descending artery or the ramus circumflex.

Exclusion criteria for both groups were unstable angina and/or non-Q wave myocardial infarction, total coronary occlusion, vessel reference diameter <3.0 mm, excessive tortuosity of the proximal vessel and the use of an ACE-inhibitor or an angiotensin II type 1 receptor blocker.

Patient characteristics are shown in Table 1. The study conforms with the principles outlined in the Declaration of Helsinki.

2.2. Tissue collection

Percutaneous coronary atherectomy was performed, both in de-novo lesions and in-stent restenosis, by means of a pullback atherectomy catheter (Arrow, Reading, PA, USA). Briefly, this 10-french compatible over-the-wire catheter is positioned beyond the lesion to treat. After the cutting blade is exposed and on-axis high-speed rotation is started, the catheter is gently withdrawn by manual pullback. This procedure allows complete circumferential cuts along the whole length of the lesion, thus providing in vivo plaque samples, suitable for histopathological analysis. In all patients, one biopsy was taken out of the culprit lesion. Coronary angiography before and after the atherectomy confirmed that the sample was taken from the lesion. After collection, the samples were immediately frozen in liquid nitrogen and stored in a −80°C freezer.

2.3. Tissue preparation and staining techniques

Frozen specimens were oriented along their longest axis in Tissue Tek. Of each specimen, 5-μm sections serially cut were mounted on glass slides. One slide was stained with haematoxylin and eosin staining and consecutive serial sections were used for immunohistochemistry. All stainings were performed in duplo. The primary antibodies we used were reactive with epitopes specific for macrophages (CD68 (clone EBM11), DAKO, Glostrup, Denmark), α-smooth muscle cells (SMA-1 (clone 1A4), DAKO), ACE (mouse antibody (clone 9B9), Chemicon, Temecula, CA, USA) and the AT1 receptor (rabbit anti-

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<thead>
<tr>
<th>Table 1</th>
<th>Patient characteristics</th>
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<tr>
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<td>Stenosis (n=19)</td>
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<tr>
<td>Age (years)</td>
<td>58</td>
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<tr>
<td>Cholesterol (mmol/l)</td>
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<td>Medication (n (%))</td>
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<tr>
<td>Aspirin</td>
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<td>Statin</td>
<td>9 (47)</td>
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<tr>
<td>Beta blocker</td>
<td>16 (84)</td>
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<tr>
<td>Calcium antagonist</td>
<td>10 (52)</td>
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<td>Nitrate</td>
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<td>Risk factors (n (%))</td>
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<tr>
<td>Smoking</td>
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<tr>
<td>Hypertension</td>
<td>11 (58)</td>
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<tr>
<td>Hyperlipidemia</td>
<td>10 (53)</td>
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<td>Time after stent placement (days (min–max))</td>
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body, Biotrend, Köln, Germany). Acetone-fixed sections were subjected to a three-step streptavidin–biotin alkaline phosphatase (AP) staining procedure. ACE and AT1 receptor antibodies were incubated overnight at 4 °C. Tris-buffered saline was used for washing between the subsequent incubation steps. Biotinylated goat anti-mouse immunoglobulin (1:200) or biotinylated goat anti-rabbit immunoglobulin (1:400) (both DAKO) were used as second step (30 min room temperature), AP-conjugated streptavidin (DAKO) as third step (1:100; 30 min room temperature). AP enzymatic activity was visualised using the Fast Red substrate system (DAKO).

Immunodoublestaining for the simultaneous identification of smooth muscle cells and macrophages was based on two primary antibodies of different IgG subclasses as described earlier [13]. Using AP as enzymatic marker, macrophages were visualised in blue with Fast Blue BB/Naphthol-AS-MX-phosphate as chromogens (Sigma, St. Louis, MO, USA). Smooth muscle cells were developed in red with peroxidase as marker enzyme and 3-amino-9-ethylcarbazole (Sigma) as chromogen.

Sections of alveolar lung tissue and infarcted myocardium served as positive controls for anti-ACE and anti-AT1 staining.

2.4. Analysis of immunostaining

A semi-quantitative score of ACE, AT1 receptor, CD68 and SMA-1 staining was applied by two independent persons (AvdW, LJW) in order to evaluate the cell type and number of cells positive in each biopsy. These persons were blinded to the patient characteristics. The weighted kappa for interobserver variability was 0.65. The semi-quantitative score we used is as follows: 0, absent; 1, few scattered cells or clusters <5 cells; 2, <10% of cells positive; 3, 10–50% of cells positive; 4, >50% of cells positive.

An immunodouble stain for smooth muscle cells and macrophages was used to facilitate the semi-quantitative scoring of macrophages and smooth muscle cells in each biopsy, and to establish the immunolocalisation of ACE and the AT1 receptor in different cell types. Anti-ACE and anti-AT1 stains were not useful for doublestaining procedures in combination with cell-specific stains (CD68 and SMA-1) because of huge differences in staining intensity.

2.5. Statistical analysis

Two-tailed t-test and chi-square tests were performed to compare continuous and categorical variables, respectively. A Spearman correlation (SPSS for Windows 10.1) was used to compare the influence of timing of the biopsy on the histological parameters. A weighted kappa coefficient (SPSS for Windows 10.1) was calculated to determine the interobserver agreement. Differences were considered significant at a level of $P<0.05$.

3. Results

Patient groups were generally well matched with respect to their baseline characteristics, although hyperlipidemia was more frequent in patients with in-stent restenosis. Neither the presence of hyperlipidemia, nor the cholesterol level were related to the immunohistochemical outcome parameters of this study (data not shown).

In biopsies from de-novo stenoses a combination of different tissue components was found, including sclerotic tissue, and lipid cores. Thrombus was found in only one biopsy. In three specimens (16%), neointimal formation was found. All but one biopsy contained both macrophages (CD68-positive) and smooth muscle cells (SMA-1-positive), but in highly variable amounts. One biopsy contained only smooth muscle cells without CD68-positive cells. Only three biopsies contained vascular smooth muscle cells (VSMCs), consistent with neointimal proliferation, i.e. stellate-shaped smooth muscle cells embedded in abundant myxoid extracellular matrix. Anti-ACE staining was observed in macrophages only. Smooth muscle cells were always ACE-negative. Anti-AT1 receptor staining was seen on both macrophages and smooth muscle cells, but macrophages showed higher staining intensity. Typical examples of biopsies from in-stent restenoses are shown in Fig. 1a–c.

In-stent restenotic lesions contained predominantly stellate-shaped VSMCs in abundant myxoid matrix, characteristic for neointimal tissue. Macrophages were either absent (11% of the tissue specimens) or present in small clusters (89%). Also in in-stent restenosis, anti-ACE staining was only seen in the macrophages, if present, whereas macrophages and all smooth muscle cells, including the stellate VSMCs in the neointimal tissue, were AT1 receptor-positive. Typical examples of biopsies from in-stent restenoses are shown in Fig. 2a–c.

Semi-quantitative analysis of immunostaining showed that in-stent restenotic lesions contained more VSMCs ($P=0.003$), but less macrophages ($P=0.002$), than de-novo lesions (Fig. 3). Moreover, in-stent restenotic lesions showed less ACE-positive cells ($P=0.02$), but slightly more AT1 receptor staining, although not significant ($P=0.16$), when compared with de-novo lesions (Fig. 3). Therefore, the overall expression of AT1 receptors in restenotic lesions appeared much more abundant than in de-novo lesions with similar clinical presentation.

The time between the stent placement and the biopsy in patients with in-stent restenosis appeared to be negatively correlated with the amount of macrophages in the in-stent restenotic plaque ($r=-0.558; P=0.013$), although the number of cases in this instance is very small (Fig. 4). As ACE was only seen in macrophages, the timing of the
biopsy is also negatively correlated to the ACE-content in the plaque ($r = -0.571; P = 0.011$).

4. Discussion

This study demonstrates the potential involvement of the renin–angiotensin system in the development of in-stent restenosis in human coronary arteries. Moreover, different patterns evolve comparing in-stent restenosis with de-novo stable coronary lesions. In de-novo stenosis, ACE was present in macrophage-rich regions in all specimens. The AT1 receptor was found on vascular smooth muscle cells (VSMCs) and macrophages. In in-stent restenosis, ACE-staining was limited to the macrophages, if present, and hence in much lower quantities than in de-novo lesions. The AT1 receptor on the other hand was more abundantly present in in-stent restenotic lesions, especially due to the large number of VSMCs.

4.1. Atherosclerosis and the renin–angiotensin system

Recent observations suggested that RAS might be involved in the occurrence of acute coronary syndromes. In fact, co-localisation of elevated ACE and angiotensin II, the RAS effector molecule in the vessel wall, in macrophage-rich areas of atherosclerotic plaques has already been demonstrated [1,2]. It is well-known that activated macrophages play a mayor role in plaque inflammation and subsequent disruption [3]. Moreover, several studies have shown the effectiveness of ACE-inhibitors in reducing the incidence of cardiovascular adverse events [14,15]. Although Powell et al. showed reduction of restenosis by ACE inhibitors in a rat model of balloon angioplasty [16], chronic ACE inhibition did not prevent restenosis after non-stent PTCA in humans [17,18].

In our study, ACE immunoreactivity appeared mainly as distinct granules in the cytoplasm of inflammatory cells and was present in all biopsies of de-novo lesions.
Fig. 2. Immunostained sections of part of an atherectomy specimen derived from an in-stent restenotic lesion. Photos are taken from an area containing neointimal tissue. (a) Immunodouble stain with anti-CD68 antibody (blue, macrophages) and anti-SMA-1 antibody (red, smooth muscle cells). The tissue composition is dominated by large amounts of spindle-shaped smooth muscle cells (red). In between is a cluster of macrophages (blue stained cells) indicated by arrow. (b) High magnification of the smooth muscle cell (VSMC)-rich neointimal tissue showing a granular type of immunostaining in red of VSMCs with anti-angiotensin II type 1 receptor antibody. Cytoplasmic staining is present in cells throughout the tissue. (c) High magnification of neointimal tissue, taken from a serial section stained with anti-angiotensin converting enzyme (ACE) antibody. Scarce cytoplasmic staining macrophages (red cells) are present. The majority of cells (in adjacent sections identified as VSMC, see (a), of which nuclei are faintly counterstained with haematoxylin, are anti-ACE negative.

The pathophysiological effects of angiotensin II, the effector molecule of the renin–angiotensin system, in the vessel wall are mediated through the AT1 receptor [10,12,19–21]. Blockade of the AT1 receptor, as well as ACE-inhibition, could be helpful in premature atherosclerosis, as already proven in animal models and humans [22,23]. We found the AT1 receptor to be present on both macrophages and VSMCs in the atherosclerotic plaque.

4.2. In-stent restenosis and the renin–angiotensin system

The process of in-stent restenosis has been described in several post-mortem studies [9,24–26]. The first step in the process is platelet deposition and aggregation, which is followed by a period of thrombus formation and inflammation, mainly at the sites of the struts. Smooth muscle cells are detected from 9–12 days after stent placement. After circa 30 days, neither thrombi nor acute inflammatory cells are found. At that time, chronic inflammation is still detectable around the struts, but the newly formed tissue responsible for restenosis development mainly consists of smooth muscle cells (96% of total plaque area) embedded in a proteoglycan-rich matrix. Such a tissue composition is characteristic for neointima formation.

Activation of the renin–angiotensin system is one of the possible mechanisms for VSMC-proliferation. Angiotensin II is able to induce proliferation of VSMCs, a key mechanism in in-stent restenosis, via stimulation of AT1 receptors in experimental models [10–12,27,28]. However, apart from a case report [29], which showed ACE activity in restenotic material from a popliteal stent, RAS contribution to human in-stent restenosis has not been elucidated yet.

In our biopsies, the immunoreactivity with anti-ACE was restricted to inflammatory cells, and present in 74% of in-stent restenotic lesions. A higher amount of macrophages (and hence of ACE-staining) was found in in-stent restenotic lesions that had the shortest interval between the stent placement and the biopsy. This indicates that also in in-stent restenosis, infiltration of inflammatory cells may
Fig. 3. Semi-quantitative scores for smooth muscle cells, macrophages, angiotensin II type 1 (AT1) receptor and angiotensin converting enzyme (ACE) in in-stent restenosis (black bars) and de-novo stenosis (grey bars).

Fig. 4. Inverse correlation between the amount of macrophages (semi-quantitative score) in the in-stent restenotic lesions and the time-interval between the placement of the stent and the biopsy ($n=19$) (mean±S.E.M.).

play a role in the RAS-pathway. The fact that we still found macrophage-rich lesions more than 30 days after stent placement is in contradiction with the above-mentioned studies about the genesis of in-stent restenosis.

The presence of the AT1 receptor on the abundantly present VSMCs in this tissue might indicate that the RAS plays a role in the growth and migration of VSMCs in in-stent restenosis. However, the role of ACE in the genesis of in-stent restenosis is still unclear. Several studies reported that the ACE-inhibitor quinapril reduces in-stent intimal hyperplasia, late luminal loss and restenosis rate [30–32]. Also, a recent retrospective study in 1598 patients suggested that ACE-inhibitors decrease late revascularisation after stent placement [33]. In the PARIS
study, on the other hand, quinapril was not able to prevent in-stent restenosis in patients with the DD genotype of ACE [34]. It remains controversial whether patients with the DD-allele of the ACE-polymorphism are especially at risk for in-stent restenosis, due to their higher ACE-activity. The D-allele has been identified as a prognostic factor for in-stent restenosis in some studies [35,36], but other larger studies did not confirm this [37–39]. Since the effects of the RAS are mediated via the AT1 receptor, blockade of the AT1 receptor could be expected to be effective in preventing in-stent restenosis. The results of the present study may endorse the outcome of a recently published study in 250 patients, which demonstrates the effectiveness of the AT1 receptor blocker valsartan in preventing in-stent restenosis after angioplasty with stent placement in type B2/C lesions [40]. Larger studies will be necessary to confirm this hypothesis.

5. Conclusions

Our results are consistent with the involvement of the renin–angiotensin system in stable coronary atherosclerosis. In de-novo lesions ACE and AT1 receptors were found on macrophages, which were present in all specimens. All VSMCs were AT1-positive, but less intense than the macrophages. In contrast, in-stent restenotic lesions consisted mainly of AT1 receptor-positive VSMCs, highly characteristic for neointimal proliferation. Macrophages were present in most biopsies, but only in small amounts. Anti-ACE and anti-AT1 receptor staining was seen on most of these macrophages. This might demonstrate the important role of the renin–angiotensin system, and especially the AT1 receptor in in-stent restenosis.

Acknowledgements

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References

[22] Hope S, Brecher P, Chobanian AV. Comparison of the effects of AT1