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Paper

Angiotensin type 2 receptor is expressed in human atherosclerotic lesions

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Abstract

Objective. Expression of the angiotensin type 2 receptor (AT_2-receptor) occurs in many animal models of atherosclerosis. However, its expression in human plaques and its functional role remains undetermined. This study examined AT_2-receptor expression in human atherosclerotic plaque and also explored its potentially important functional role in atherosclerosis.

Material and methods. We analysed carotid atherosclerotic plaques obtained from 14 Caucasian patients who had previously undergone endarterectomy for symptomatic carotid artery stenosis. Half of all subjects received treatment with an angiotensin receptor blocker (ARB) (n=7); the remaining subjects received no intervention in the renin-angiotensin system (n=7). Immunohistochemistry measured tissue expression of smooth muscle cells (α-actin), macrophages (CD68 antibody), collagen (picro-sirius), and AT_2-receptor (AT_2-receptor antibody).

Results. AT_2-receptor expression occurred consistently in all specimens. Although cellular localisation varied, AT_2-receptor expression levels correlated with macrophage levels ($p<0.01$). Compared to conventional treatment, ongoing ARB treatment affected neither AT_2-receptor levels nor plaque composition.

Conclusions. AT_2-receptor is expressed in human atherosclerotic plaque. Furthermore, we detected no functionally important role of AT_2-receptor expression and found no evidence that ARB treatment regulates AT_2-receptor expression.

Introduction

Angiotensin (Ang) II, the major effector peptide of the renin-angiotensin system (RAS), is a pro-atherogenic agent.[1,2] Activation of angiotensin type 1 (AT_1) receptor likely affects both the extent and composition of atherosclerotic plaque.[3] However, Ang II also activates its type 2 (AT_2) receptor. The expression pattern, function, and importance of AT_2-receptor remain unclear.[4] Despite contradictory reports,[5,6] the actions of AT_2-receptor likely oppose those of AT_1-receptor, slowing atherosclerosis development and promoting a more stable plaque phenotype.

Previously, we and others demonstrated abundant expression of AT_2-receptor in murine atherosclerotic lesions.[7-9] AT_2-receptor expression increases in hypertensive mice and also in mice treated with angiotensin receptor blocker (ARB).[8] Moreover, absence of AT_2-receptor increases macrophages and smooth muscle cells (SMC) and augments collagen levels, possibly due to AT_2-receptors effect in diminishing proliferation and amplifying apoptosis of lesion cells.[9] Such data suggest that AT_2-receptor might confer stability to the atherosclerotic plaque.

In humans, AT_2-receptor occurs in the resistance arteries of hypertensive subjects. Savoia *et al.*,[10] showed that ARB treatment (one year) increases AT_2-receptor expression and improves vascular function. However, earlier studies did not examine the role of AT_2-receptor in human atherosclerotic lesions. Therefore, our study sought to determine whether AT_2-receptor expression occurs in such lesions and also identify which cell type(s) expresses AT_2-receptor. We further investigated whether pharmacological interventions in the RAS influence AT_2-receptor expression and whether they simultaneously modify indicators of lesion stability such as collagen content, SMC expression, and macrophages.

Material and methods

Study population

We obtained carotid atherosclerotic plaques from 14 Caucasian patients enrolled in the Göteborg Atheroma Study Group. All patients had undergone endarterectomy previously at Sahlgrenska University Hospital for symptomatic carotid artery stenosis, and all patients completed a medical history questionnaire. We sorted individuals into two groups. Patients in one group received ARB treatment (n=7); patients in the second group (n=7) received neither ARB nor angiotensin-converting enzyme (ACE) inhibitor treatment.

Immunohistochemistry

After dividing carotid endarterectomy specimens into sections (3 mm thick), we fixed them in formalin for 24-hours, and embedded them in paraffin. Sections (4 µm thick) corresponding to

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Table 1
Characteristics of patients with symptomatic carotid stenosis (n=14).

Variable	ARB therapy	
	Yes (n=7)	No (n=7)
Women, n (%)	1 (14)	2 (29)
Age, years	69±4	67±8
Clinical event, type		
Stroke, n (%)	5 (56)	5 (71)
TIA, n (%)	2 (22)	2 (29)
Amaurosis fugax, n (%)	2 (22)	0
Time since clinical event, days (median, range)	113 (42-172)	82 (56-102)
Previous MI, n (%)	1 (14)	1 (14)
Hypertension, n (%)	7 (100)	5 (71)
Diabetes, n (%)	2 (29)	3 (43)
Current smoker, n (%)	0 (0)	0 (0)
Previous smoker, n (%)	3 (43)	6 (87)
Statin therapy, n (%)	6 (86)	7 (78)
Blood pressure, mmHg		
Systolic	148±17	144±17
Diastolic	79±7	73±8
Serum HDL (mmol/L)	1.0±0.2	1.0±0.2
Serum total cholesterol (mmol/L)	4.5±1.3	4.2±0.5
Serum LDL cholesterol (mmol/L)	2.5±0.9	2.6±0.5
Serum triglycerides (mmol/L)	2.1±0.8	1.4±0.6
BMI	30.0±6.4	27.1±2.3

Key: Data expressed as mean±SD. BMI = body mass index; HDL = high-density lipoprotein; LDL = low-density lipoprotein; MI = myocardial infarction; TIA = transient ischaemic attack.

the internal carotid artery, 9–15 mm from the bifurcation, from each endarterectomy were used for immunohistochemistry. Following de-paraffinisation antigens were unmasked with target retrieval solution (DAKO Cytomation, Buckinghamshire, UK). Using a standard LSAB+ System-HRP Kit (DAKO) according to manufacturer's instructions, we performed immunohistochemical staining for macrophage marker CD68 (Novocastra Laboratories Inc., Newcastle, UK) and SMC marker anti-muscle actin (Enzo Diagnostics. Inc, Farmingdale, NY). As a negative control, we substituted primary antibody with universal negative control reagent. Picro-sirius red staining was used for collagen expression. Immunostaining for AT_2-receptor followed a protocol described previously.[8] We incubated specimens overnight with primary AT_2-receptor antibody (#sc-7421, Santa Cruz Biotechnology, California, USA). To further verify the specificity of the AT_2-receptor antibody, we mixed blocking peptide (#sc-7421P, Santa Cruz) with primary antibody (1:20) overnight at +4°C, totally abolishing AT_2-receptor staining. BioPix software (version 1.6.1, Holmdahl BioTech, Göteborg, Sweden) automatically traced positive staining for CD68, collagen, and AT_2-receptor. In addition, we calculated average positive staining for two serially sectioned samples. Positive staining area is expressed as percentage of total lesion area. We also stained sections with hematoxylin-eosin and categorised them according to American Heart Association (AHA) classification[11] and recorded all occurrences of plaque rupture. AHA classification and plaque rupture were assessed in 68 sections for each group, these sections were matched for location for each group and included sections from the bulb, the common-, the external-, and the internal-carotid artery.

Statistics

We analysed population data using SPSS 12.0.1 (SPSS Inc., Chicago, IL). Data for AT_2-receptor, SMC, collagen, CD68 were analysed using the

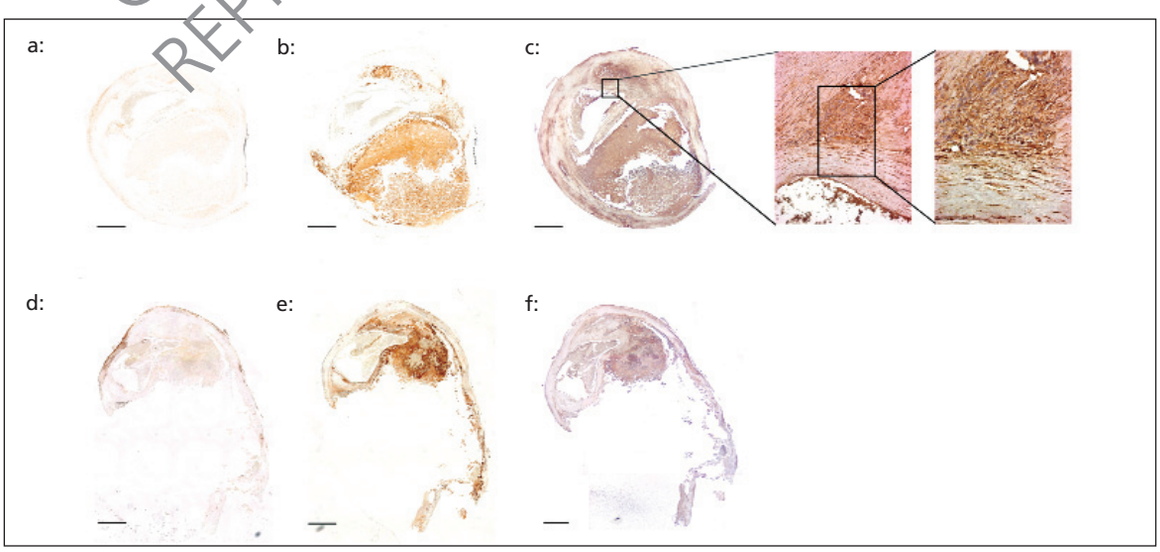

Figure 1
Immunostaining for smooth muscle cell marker α-actin (**a**, **d**), macrophage marker CD68 (**b**, **e**) and AT_2-receptor (**c**, **f**) in patients with (**a-c**) or without (**d-f**) interventions in the RAS. Scale bar represents 1 mm. Inserts display 20x and 40x magnification.
AT_2-receptor = angiotensin type 2 receptor; RAS = renin angiotensin system.

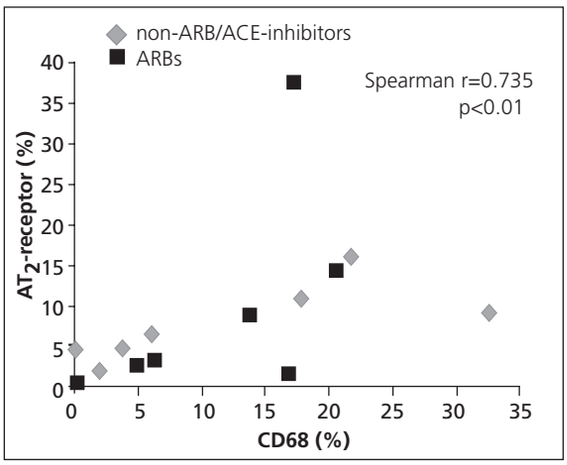

Figure 2
Positive correlation between AT_2-receptor and macrophage marker CD68 in human carotid atherosclerotic plaques. Spearman r=0.735, p<0.01. ACE = angiotensin-converting enzyme; ARB = angiotensin receptor blocker; AT_2-receptor = angiotensin type 2 receptor.

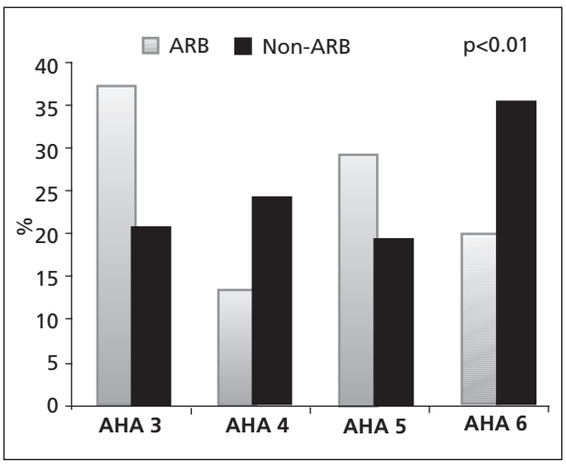

Figure 3
Description of plaque severity according to the American Heart Association classification in patients receiving (n=7) or not receiving (n=7) ARB. p<0.01. AHA = American Heart Association; ARB = angiotensin receptor blocker.

Mann-Whitney U-test, and correlations that did not fulfil the criteria for parametric testing were analysed using the Spearman correlation test (SPSS). All data are expressed as mean±SEM, except for patient characteristics in table 1, which is expressed as mean±SD. A value of $p<0.05$ was considered statistically significant.

Results

Table 1 shows the characteristics of all study participants. We observed positive staining for AT_2-receptor in all sections (representative cases shown in figure 1). Although we detected a positive correlation between AT_2-receptor expression and macrophage marker CD68 (Spearman r=0.735, p<0.01, figure 2), we found no similar correlation for SMC or collagen (Spearman r=0.20, p=0.51, Spearman r=0.20, p=0.48, respectively).

ARB treatment did not affect AT_2-receptor expression compared to the untreated group (table 2), and we observed no differences in CD68-positive macrophages, SMC, or collagen (table 2, figure 1). According to AHA classification, patients receiving ARB treatment had less complicated plaques compared with patients who received no such treatment (p<0.01, figure 3). We observed plaque rupture in 20% of ARB-treated patients versus 36% in patients without treatment (p<0.01).

Discussion

The present study shows for the first time that AT_2-receptor protein expression occurs in human atherosclerotic lesions, thus amplifying earlier descriptions of AT_2-receptor expression in mouse[7,9] and rabbit[12] lesions. AT_2-receptor is expressed abundantly in developing tissue but only rarely in healthy tissue from mature individuals.[5] Since inflammatory cytokines induce AT_2-receptor expression[13] and re-expression of AT_2-receptor follows vascular injury that elicits an inflammatory response,[14] greatly increased levels of AT_2-receptor in atherosclerotic lesions were unsurprising.[7-9]

Despite our findings, the functional importance of AT_2-receptor expression remains unclear. Although earlier studies indicated that

Table 2
Quantification of AT_2-receptor, CD68 positive macrophages, SMC and collagen in atherosclerotic lesions obtained from patients with symptomatic carotid stenosis.

	AT_2-receptor (%)	Macrophages (%)	SMC (%)	Collagen (%)
RAS intervention	9.6±5.0	11.4±2.9	4.7±2.3	14.4±1.4
Non-RAS intervention	7.8±1.8	12.0±4.6	0.6±0.3	18.7±4.5
p =	0.535	1.000	0.073	0.535

Key: Data is expressed as percentage of total lesion area and presented as mean±SEM. AT_2-receptor = angiotensin type 2 receptor; RAS = renin-angiotensin system; SMC = smooth muscle cells.

pharmacological disruption of AT_2-receptor signalling leads to accelerated atherosclerosis,[15] others failed to observe such an effect.[7] However, if AT_2-receptor does not affect the extent of atherosclerosis, it may alter the plaque's cellular composition. Indeed, AT_2-receptor binding activates nitric oxide signalling,[16] a recognised pathway for anti-proliferation and anti-inflammation.[17] In atherosclerotic mouse lesions, disrupted AT_2-receptor signalling increases the level of macrophages, SMC, and collagen.[9] Moreover, plaques from apolipoprotein E-/- mice lacking AT_2-receptor have reduced TUNEL staining and increased incorporation of bromodeoxyuridine, possibly due to AT_2-receptor's ability to reduce plaque cellularity by affecting apoptosis and proliferation.[9]

Our cross-sectional material made speculation regarding the functional importance of AT_2-receptor expression difficult. Nonetheless, we attempted to address this issue by quantifying plaque composition and AT_2-receptor expression separately in patients treated previously with ARB or in patients without ARB/ACE-inhibitor treatment. ARB treatment increases angiotensin levels significantly, and also might act on unopposed AT_2-receptor,[18] thus altering plaque characteristics. Others have shown that ARB treatment stabilises human atherosclerotic plaques.[19] However, although a trend was seen, the data presented here showed no significant alteration in plaque composition between ARB- and non-ARB/ACE-inhibitors-treated patients. Interestingly, although ARB treatment in resistance arteries of hypertensive and diabetic humans increases AT_2-receptor expression,[10] ARB treatment did not increase AT_2-receptor expression in the current study (p=0.535). Thus, our results do not support the hypothesis that ARB treatment increases AT_2-receptor expression in human plaques.

AT_2-receptor expression localises generally in SMC,[14] endothelial cells, and fibroblasts but only rarely in immune competent cells.[20] However, most AT_2-receptor expression in plaques taken from cholesterol-fed New Zealand white rabbits occurred in SMC and macrophages.[9,12] Unfortunately, our study did not show unequivocally the cellular origin of AT_2-receptor expression. A heterogeneous expression profile was evident when we compared the staining of adjacent sections. In some areas of the plaque, intense macrophage staining co-localised with similarly intense AT_2-receptor staining. However, this pattern was missing in other areas. Following quantification of the different stainings, we identified a significant correlation between AT_2-receptor and macrophage staining, suggesting that inflammatory activity in the plaque might stimulate AT_2-receptor expression.[13] We observed no apparent co-localisation of AT_2-receptor with SMC.

Study limitations

We examined the impact of ARB treatment on AT_2-receptor expression in plaques in a non-randomised study of limited size. In addition, our lack of information regarding the duration of ARB treatment and whether patients in the control group previously received interventions in the RAS presented limitations when we compared our results to Savoia *et al.*[10] and Cipollone *et al.*[21] They showed that one year and four months of ARB treatment affected plaque stability and expression of AT_2-receptor respectively. Furthermore, due to the limited patient size, it is unclear as to whether the presence of hypertension and possibly type 2 diabetes is associated with a change in AT_2-receptor expression. Moreover, a larger sample size might have revealed differences in smooth muscle cell content and thereby a possible functional role for the AT_2-receptor. However, the patho-physiological role of the AT_2-receptor is still largely unclear.

In conclusion, AT_2-receptor is expressed in human atherosclerotic plaque. Although the correlation between AT_2-receptor and macrophage staining suggests that inflammatory activity affects expression patterns, we determined that AT_2-receptor expression plays no functionally important role in plaque. An important caveat in the current study is the limited sample size. However, we found no evidence that ARB treatment regulates the expression of AT_2-receptor.

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