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Persistent and selective upregulation of renin–angiotensin system in circulating T lymphocytes in unstable angina

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Abstract

Introduction: Unstable angina is associated with an acute systemic inflammatory reaction and circulating T lymphocytes are activated. We investigated whether in unstable angina with marked immune system activation a selective upregulation of the circulating T-cell renin–angiotensin system, modulated by angiotensin II, could occur.

Methods: We studied 13 unstable angina patients, 10 patients with stable angina and 10 healthy subjects. After T-lymphocyte isolation, mRNAs for angiotensin-converting enzyme (ACE) and angiotensin type 1 receptor (AT1-R) were quantified at baseline and after angiotensin II stimulation. ACE activity in cell pellet and supernatant and angiotensin II cell content were measured.

Results: Plasma renin activity was similar in controls, stable and unstable angina patients. At baseline ACE and AT1-R mRNA levels were higher ($P<0.05$) in T cells from unstable angina patients than in T cells from stable angina patients and controls, and further increased after angiotensin II addition to cultured T cells. ACE activity of unstable angina T cells was significantly higher than that of T cells from controls and stable angina patients. Only in T cells from unstable angina patients did angiotensin II stimulation cause the almost complete release of ACE activity in the supernatant.

Conclusions: The circulating T-cell-based renin–angiotensin system from unstable angina patients was selectively upregulated. In vivo unstable angina T cells could locally increase angiotensin II concentration in tissues where they migrate independently of the circulating renin–angiotensin system.

Keywords

Angiotensin-converting enzyme, angiotensin II, T cell, unstable angina, renin–angiotensin system

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Introduction

The renin–angiotensin system (RAS) is well known to play an important role in the initiation and amplification of atherosclerosis damage that clinically results in cardiovascular disease. In previous studies[1] we demonstrated that in patients with unstable angina the cardiac RAS is activated and sustains an increased de novo production of cardiac angiotensin (Ang) II. Upregulated cardiac RAS participates in the coronary microvessel inflammation of unstable angina and strengthens the immunomediated component of myocardial inflammation. Indeed, in unstable angina myocardium-positive immunostaining for angiotensin-converting enzyme (ACE) co-localised with T cells and endothelial cells. In agreement with this finding, experimental and human studies showed that Ang II may be produced by T cells that are fully equipped with RAS components and specifically can express the gene for key components of the RAS, such as ACE and angiotensinogen.[2–4] According to these findings, Ang II acts on and is produced by inflammatory cells,[5] can affect T-cell behaviour by angiotensin type 1 receptor (AT1-R) and induce cellular interferon-gamma (IFN-γ) secretion and interleukin 2 production.[6] T-cell RAS is functionally

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Table 1. Clinical characteristics of controls, stable and unstable angina patients.

Characteristics	Controls	Stable angina patients	Unstable angina patients	ANOVA
	(*n*=5)	(*n*=10)	(*n*=13)	
Men/women	3/2	6/4	8/5	0.938
Age (years)	46±9	56+11	63±6	*P*<0.01
Smokers (%)	1(20)	6(60)	8(67)	*P*<0.01
Hypertension (%)	0	7(70)	11(84)	*P*<0.01
Fasting glucose (mg/dl)	84±9	92±5	89±10	ns
Total cholesterol (mg/dl)	188±24	191±40	193±45	ns
HDL-cholesterol (mg/dl)	52±11	46±17	54±16	ns
Creatinine (mg/dl)	0.79±0.09	0.89±0.27 0.	89±0.15	ns
LVM (g/m^2)	106.3±17.3	107.2±20.4	106.9±18.9	ns
LVEF (%)	60.4±5.2	55.1±4.6	54.9±5.3	ns
hs-CRP (mg/dl)	1.1±0.5	1.2±0.7	4.8±1.9	*P*<0.01

ANOVA: analysis of variance; HDL: high-density lipoprotein; LVM: left ventricular mass; LVEF: left ventricular ejection fraction; hs-CRP: high-sensitivity C-reactive protein.

autonomous from circulating and various tissue-based RAS and can synthesise Ang II that acts as a positive feedback loop on inflammatory cells to amplify the inflammatory reaction further.[7–9] In our previous paper we showed that in hypertensive patients with low grade inflammation the T-cell RAS response to Ang II is amplified in comparison with T cells from controls and hypertensive individuals without inflammation. Our findings gave strong support to the hypothesis that T-cell RAS activity was strictly related to inflammatory lymphocyte activation. In unstable angina patients, circulating T cells are activated and sustain the acute systemic inflammation that precipitates plaque instability. Serum high-sensitivity C-reactive protein (hs-CRP) is a sensitive indicator of inflammation, which is closely related to the progress of plaque and markedly increases in acute coronary syndrome (ACS). The increase in hs-CRP is slight or absent in patients with stable coronary plaques. Up to now, no data are available about the behaviour of circulating T-cell RAS of patients with ACS or stable angina. This study was aimed at investigating whether an activation of cell-based RAS could be present in circulating T cells from patients with unstable or stable angina with increased or normal hs-CPR levels. We also investigated whether in vitro the addition of Ang II could stimulate or inhibit cultured T-cell RAS from unstable or stable angina patients.

Materials and methods

We examined 13 patients with unstable angina in classes IIB (*n*=5) and IIIB (*n*=8) and 10 patients with stable angina in Canadian classes II (*n*=4) and III (*n*=6), who had been admitted to the cardiological intensive therapy unit of Careggi Teaching Hospital, Florence, Italy.

We excluded those patients on ACE inhibitors and/or Ang II receptor antagonists and those with acute or chronic diseases accompanied by evidence of circulating RAS activation. Ten subjects in apparent good health comparable for age and sex to unstable and stable angina patients formed the control group.

The protocol of the study complies with the principle of the Helsinki declaration and was approved by the ethical committee of our institution. All patients gave written informed consent to participate and to have blood samples taken for the study.

The main demographic and clinical characteristics of enrolled patients are shown in Table 1.

Experimental procedures

T-lymphocyte isolation

Venous blood was drawn from the antecubital vein, collected in a sterile flask containing 3.8% sodium citrate (9/1, vol/vol); the method for T-lymphocyte isolation was detailed in our previous report.6 Magnetic beads conjugated with antihuman CD 3 (Miltenyi Biotec, Germany) were used for the positive selection of T lymphocytes. In each incubation experiment mRNA expression of ACE, AT1-R, IFN-γ and ACE activity and Ang II concentrations were evaluated in T-cell pellets. ACE activity was also investigated in the supernatants, because ACE can be shed by T cells.[6] To study the effect of stimulation by Ang II on ACE and AT1-R T-cell gene expression, ACE activity and Ang II concentrations, T lymphocytes had been cultured in a cell incubator in humidified atmosphere with 5% carbon dioxide for 6, 18 or 24 hours, with and without the addition of $10^{-13.5}$ M/ml Ang II to the culture medium.

Real-time polymerase chain reaction for ACE gene, RT–PCR analysis for AT1-R and IFN-γ genes

Isolation of total RNA was performed as previously described.[6] FAM-labelled probes and primers for ACE

Table 2. Primer and probe sequences in real-time PCR; primer sequences and PCR conditions in RT–PCR.

Target	Primer sequence	Product size	Annealing, C°	Cycle, *n*
ACE	Probe 5′-ACC ACG ACG GCGGAG GGG-3′			
β-actin	Forward 5′-TGA GCG CGG CTA CAG CTT-3′ Reverse 5′-TCC TTA ATG TCA CGC ACG ATT T-3′ Probe Hs00174179_m1		52	
GAPDH	Forward 5′-TGAAGGTCGGAGTCAACGGA-3′ Reverse 5′-CATGTGGGCCATGAGGTCCA-3′	983 pb	60	35
AT1-R	Forward 5′-GATGATTGTCCCAAAGCTGG-3′ Reverse 5′-TAGGTAATTGCCAAAGGGCC-3′	255 pb	53	37
IFN-γ	Forward 5′-AGTTATATCTTGGCTTTTCA-3′ Reverse 5′-ACCGAATAATTAGTCAGCTT-3′	356 pb	53	30

ACE: angiotensin-converting enzyme; AT1-R: angiotensin type 1 receptor; GAPDH: glyceraldeyde-3-phosphate deydrogenase; IFN-γ: interferon gamma; PCR: polymerase chain reaction; RT–PCR : reverse transcriptase polymerase chain reaction.

(ACE Hs00174179_m1; Applied Biosystems, Foster City, CA, USA) and for the housekeeping gene β-actin (Invitrogen, Milan, Italy) were used. ACE gene expression was calculated as $2^{\Delta Ct}$ ($\Delta C_t = C_t$ of the target gene minus C_t of β-actin); mRNAs for AT1-R and IFN-γ were semi-quantified versus the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by reverse transcriptase polymerase chain reaction (RT–PCR) (Applied Biosystems) using specific primers at annealing temperature and cycle number as reported in Table 2.

Measurement of pellet and supernatant ACE activity

T-cell ACE activity was measured by the quantification of hippuric acid, produced during the enzymatic reaction of ACE on a synthetic substrate hippuryl–histidyl–leucine and assayed by high-pressure liquid chromatography (HPLC) analysis using a spectrophotometric detector at 228 nm absorbance as previously described.[6] Ang II levels were expressed as fmol/mg proteins.

Measurement of plasma renin activity, Ang II and hs-CRP

Plasma renin activity (PRA) measurement was performed with a commercial kit (Sorin Biomedica, Saluggia, Vicenza, Italy). Ang II levels were determined by radio-immunoassay after C18 Sep-Pak cartridge extraction and HPLC separation and were expressed as fmol/mg proteins.[6] hs-CRP was measured by a commercial kit (Beckman, Brea, CA, USA) with the lowest detection point of 0.1 mg/dl.

Statistical analysis

Data have been stored in a dedicated database and analysed by IBM-SPSS 20 for Windows statistical software (IBM-SPSS Inc., USA). Variables were reported as mean±SD and frequencies (percentage). Between-groups gender comparison was assessed with the chi-square test. Within-groups comparisons (data with and without Ang II stimulus) were made by paired Student's t-test analysis. In order to analyse differences in ACE and AT1-R gene expression and ACE activity throughout the time course among the three groups, an analysis of variance (ANOVA) was used. Statistical significance was taken as $P<0.05$ for all calculations.

Results

PRA and hs-CRP in controls, stable and unstable angina patients

In control subjects, PRA in venous blood was 10.5±1.5 pmol/L per minute (range 4.5–15.2 pmol/L per minute). PRA levels in stable and unstable angina patients were 9.8±1.2 pmol/L per minute (range 4.4–14.7 pmol/L per minute) and 10.4±1.7 pmol/L per minute (range 4.0–15.8 pmol/L per minute), respectively, without significant differences among groups.

hs-CRP was significantly higher in unstable in stable angina patients and controls (Table 1).

T-cell IFN-γ gene expression with and without Ang II stimulation

T cells from UA patients showed an IFN-γ gene expression significantly higher ($P<0.01$) than those of T cells from controls and stable angina patients (densitometric ratio mRNA/mRNA GAPDH 1.37±0.29 vs. 0.71±0.16 and 0.68±0.13, respectively) (Figure 3).

The addition of Ang II to the T-cell culture was associated with a significant increase in control and stable angina T-cell IFN-γ mRNA levels, which peaked at the 24th hour without differences between the two groups. Twenty-four hours after Ang II stimulation the increase in IFN-γ mRNA levels in unstable angina T cells was significantly higher ($P<0.01$) than those observed in T cells from controls and

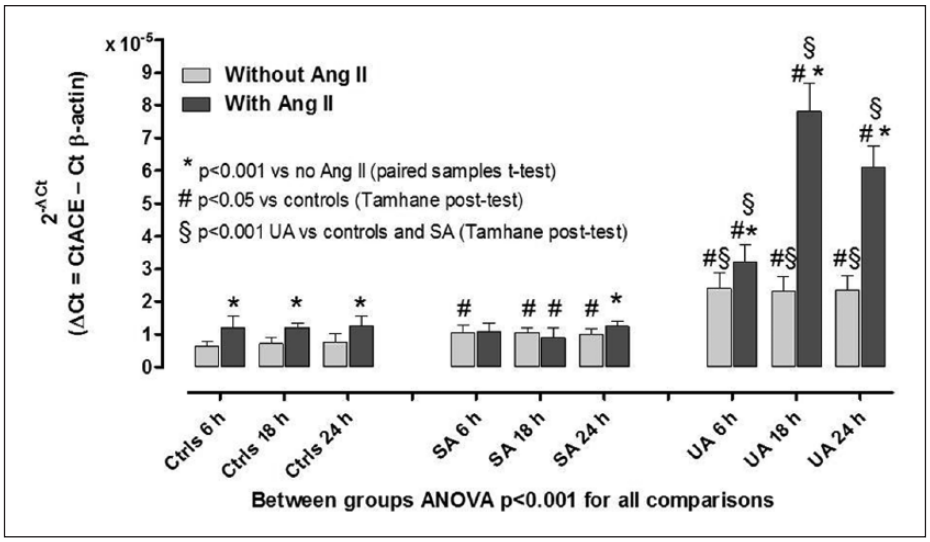

Figure 1. T-lymphocyte angiotensin-converting enzyme (ACE) gene expression with and without $10^{-13.5}$ M/ml angiotensin (Ang) II stimulation in controls, stable angina (SA) and unstable angina (UA) patients. ANOVA: analysis of variance.

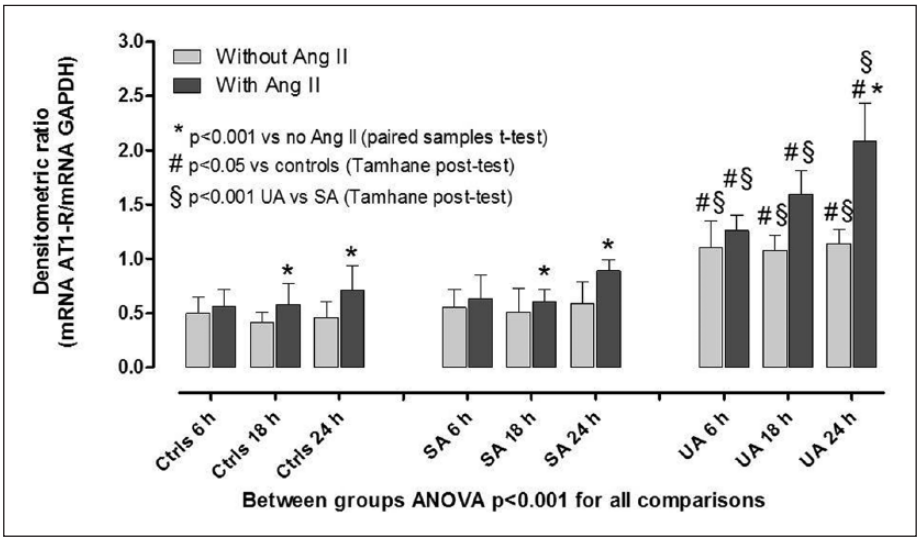

Figure 2. T-lymphocyte angiotensin type I receptor (AT1-R) gene expression with and without $10^{-13.5}$ M/ml angiotensin (Ang) II stimulation in controls, stable angina (SA) and unstable angina (UA) patients. ANOVA: analysis of variance.

stable angina patients (densitometric ratio mRNA IFN-γ/mRNA GAPDH 2.53±0.41 vs. 1.35±0.26 and 1.48±0.19, respectively).

T-cell ACE and AT1-R gene expression from controls, stable and unstable angina patients with and without Ang II stimulation

In T cells from controls ACE gene expression was detectable at baseline and did not significantly change at 18 and 24 hours of cell incubation. The addition of $10^{-13.5}$ M/ml Ang II to the culture medium caused a significant (P<0.01 vs. no Ang II stimulus) increase in the T-cell ACE mRNA level at the 24th hour of incubation (Figure 1). Similarly, T-cell AT1-R gene expression was detectable under baseline conditions and did not significantly modify at 18 and 24 hours of incubation without the addition of Ang II, but significantly increased (P<0.01 vs. no Ang II stimulus) at the 24th hour when T lymphocytes were stimulated by Ang II (Figure 2).

In stable angina patients T-cell ACE gene expression was detectable at baseline, did not change throughout cell incubation and was significantly higher (P<0.05) than that observed in the control group. The addition of Ang II to the culture medium caused a significant (P<0.01 vs, no Ang II stimulus) increase in the T-cell ACE mRNA level that

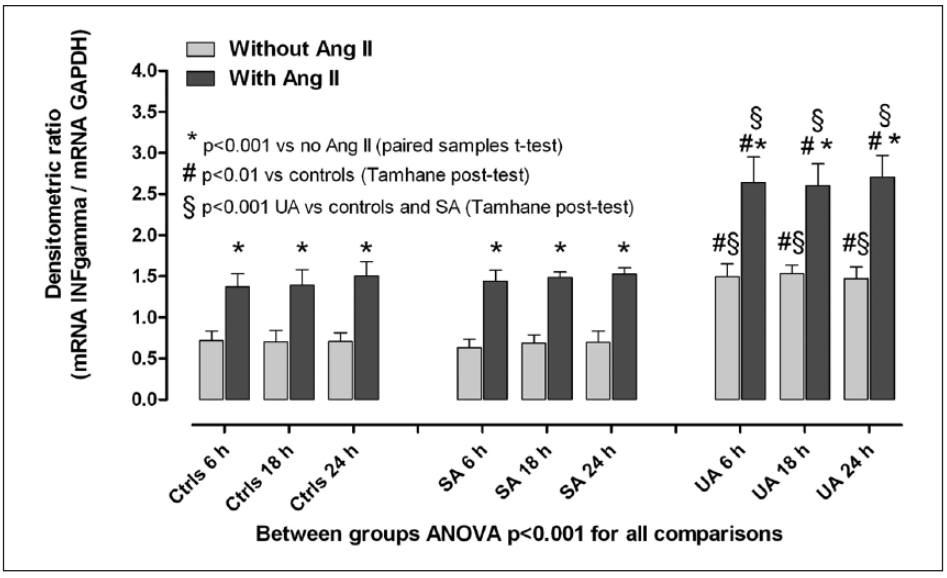

Figure 3. T-lymphocyte interferon (IFN)-γ gene expression with and without $10^{-13.5}$ M/ml angiotensin (Ang) II stimulation in controls, stable angina (SA) and unstable angina (SA) patients. ANOVA: analysis of variance.

peaked at the 24th hour (Figure 1). Similarly, T-cell AT1-R gene expression was detectable under baseline, did not significantly modify in the absence of Ang II stimulus but significantly increased (P<0.01 vs. no Ang II stimulus) 24 hours after Ang II addition (Figure 2). The pattern of T-cell ACE and AT1-R gene expression did not differ between controls and stable angina patients.

At baseline unstable angina T cells showed significantly higher (P<0.001) ACE and AT1-R mRNA levels than those expressed by T cells from controls and stable angina patients. Unstable angina T cells, ACE and AT1-R mRNA levels further increased (P<0.001 vs. no Ang II stimulation) after Ang II addition; the highest peak value for ACE mRNA was observed at the 18th hour and for AT1-R gene at the 24th hour.

ACE activity and Ang II concentration in T cells from controls, stable and unstable angina patients

At baseline, ACE activity was detectable and similar in T cells from controls and stable angina patients; Ang II stimulation induced in both groups a significant increase in pellet ACE activity that peaked at the 24th hour and was associated with the release of about 35% of total activity into the supernatant without differences between the two groups (Table 3).

Unstable angina T-cell pellet ACE activity was significantly higher (P<0.01) than that found in T cells from control subjects and stable angina patients and further increased after Ang II stimulation that determined an almost complete release of enzymatic activity in the supernatant (Table 3).

The Ang II concentration in the pellet of T cells from controls and stable angina patients was detectable under baseline conditions, did not change throughout the 24 hours of incubation, but significantly increased at the 24th hour after Ang II addition. In cultured unstable angina T cells the Ang II concentration was significantly higher (P<0.01) than that assayed in controls and stable angina patients, both at baseline and after Ang II stimulation (Table 3).

Discussion

Our data show that in unstable angina patients with high hs-CRP levels but not in stable angina patients with low hs-CRP levels, circulating T-cell RAS was upregulated under baseline conditions, independently of Ang II stimulation. The exaggerated upregulation of unstable angina T-cell RAS was mainly sustained by the high levels of ACE gene expression and resulted in a marked increase in T-cell ACE activity and Ang II concentration associated with an increased baseline AT1-R gene expression. In T cells from stable angina patients a slight increase in ACE gene expression was found, but it did not result in higher ACE activity. Stimulation of unstable angina cultured T-cell RAS by Ang II further amplified the T-cell ACE and AT1-R gene activation and resulted in a higher intracellular Ang II concentration and in the almost complete release of T-cell ACE activity in the supernatant. These findings strongly suggest that in vivo in unstable angina patients T-cell RAS could increase the local Ang II concentration inside plaques or in microvessels of left ventricular myocardium where they migrate. T cells are involved in the process of endothelial dysfunction contributing to plaque destabilisation[10] and the

Table 3. ACE activity (pellet and surnatant) and Ang II concentration (pellet) in T lymphocytes of controls, stable and unstable angina patients in the presence of $10^{-13.5}$ M Ang II concentration in cell culture medium.

	Baseline	24 h	24 h + Ang II	
ACE μU/10^6 cells				
CTL	Pellet	6.8±2.1	6.1±1.7	10.4±2.9*
	Surnatant		1.0±0.4	4.1±1.6#
SA	Pellet	6.5±1.8	7.0±2.3	11.1±2.4*
	Surnatant		0.8±0.5	4.7±1.0#
UAP	Pellet	26.7±8.9	28.1±10.2	18.5±3.8*#
	Surnatant		22.7±9.5	47.1±10.4#
Ang II fmol/mg proteins				
CTL		176.5±17.9	180.2±13.4	257.2±18.1*#
SA		184.1±19.0	177.8±10.6	249.2±19.4*#
UAP		408.0±37.2	373.1±39.6	396.4±30.7

*P<0.05 vs. baseline; #P<0.05 vs. 24 hours.
Between the groups studied : at analysis of variable unstable angina P<0.001 vs. controls and stable angina for pellet and supernatant angiotensin-converting enzyme (ACE) activity through the entire time course.
Ang: angiotensin; CTL: control; SA: stable angina; UAP: unstable angina patients.

increased production of Ang II by T cells could play a major role in inducing endothelial damage. In a previous study we demonstrated that the cardiac RAS acts locally and sustains generalised inflammation in the smaller coronary vessels of patients with unstable angina[11] by the novo production of Ang II; in unstable angina patients the heart can contribute Ang II to the circulation and play a role in maintaining systemic inflammation of unstable angina. The demonstration of the upregulation of circulating T-cell RAS in unstable angina patients shows that T cells are actively involved in the microvessel inflammation and can further amplify the systemic and cardiac activation of the immune system, also by the cell-based synthesis of Ang II. In agreement with our data, previous research indicated that inflammation and immune cell infiltration are associated with cardiovascular diseases and play a major role in the development of atherosclerosis.[12,13] Clinical trials have shown that early administered ACE inhibition improves prognosis in patients with myocardial infarction and unstable angina[14] and indirectly suggests a role of RAS in ACS. The Heart Outcomes Prevention Evaluation study showed that an ACE inhibitor reduced cardiovascular death, myocardial infarction[15] and the need for revascularisation after stenting, and reduced subsequent events in patients with angina pectoris.[16] In patients with non-ST segment elevation myocardial infarction, enalapril and ilbesartan treatment started at admission or at discharge was associated with a decrease in the levels of markers of inflammation, endothelial dysfunction and ischaemia at 60 days. As a whole, these findings support that RAS activation is involved in the inflammatory stress associated with atherosclerotic damage of coronary vessels. Ang II is one of the most potent mitogens that induce monocytes entering into vessel walls to cause inflammatory response in vascular smooth muscle cells,[17] and might be associated with the development of atherosclerosis via regulating specific T-cell phenotypes.[18]

In the past few years, a lymphocytic-based RAS was shown in humans that operates as an autonomous source of Ang II, independent of circulating and tissue RAS.[19–21] According to these findings, lymphocytes are endowed not only with ACE activity, as first reported,[20] but also with all RAS genes necessary for Ang II synthesis and can autonomously modulate the intracellular Ang II concentration. Under physiological conditions, Ang II upregulates lymphocytic RAS through AT1-Rs.[6] In our previous paper about hypertensive patients with low grade inflammation, the T-cell RAS response to Ang II is amplified in comparison with T cells from controls and hypertensive patients with hs-CRP less than 2mg/dl, suggesting that T-cell RAS activity is strictly related to inflammatory lymphocyte activation.[21] In T cells from hypertensive patients with low grade inflammation, no activation of T-cell RAS was found under baseline conditions without Ang II stimulus. Remarkably, in T cells from unstable angina patients with high hs-CRP levels, T-cell RAS was activated also under baseline conditions independently of Ang II stimulus. The amount of Ang II synthesised by unstable angina T cells could not modify Ang II concentration in plasma, as shown by normal PRA and Ang I and II levels found in this and previous work,[22] but they could affect the local Ang II concentration in tissues where they migrate. In vitro we showed that Ang II stimulation caused the almost complete release of ACE activity by T cells in supernatant; this finding strongly supports the concept that the upregulation of unstable angina T cells is aimed at increasing extra (plaques and coronary microvessels) more than intra T-cell Ang II concentrations.

The in vitro marked release of ACE enzymatic activity into the supernatant by cultured T cells from unstable angina patients could translate in vivo into the local activation of other cells endowed with AT1-R, such as endothelial cells and monocytes localised inside coronary plaques or coronary microvessels.[23]

A role for inflammatory mediators during the evolution of ACS is indicated by the widespread coronary inflammation found during unstable angina, throughout the entire coronary artery bed, and not only in the artery containing the culprit lesion shown by us and by other groups.[3,4] The pathophysiological mechanisms underlying a worse evolution of ACS patients with systemic inflammation are still under debate. Our findings show that in unstable angina the activation of T cells is associated with the selective upregulation of T-cell RAS. Unstable angina T cells can be the key step between inflammation and RAS activation that can reciprocally potentiate according to a positive feedback loop.

According to our findings the role of culprit lesion played by unstable plaques could be mediated by the increased Ang II production by activated circulating T cells migrated inside those plaques.[24,25]

Study limitations

ACE and Ang II can induce or suppress specific classes of T cells. In an experimental model proinflammatory T helper (Th)1 and Th17 cell induction was associated with increased Ang II in both CD4 T cells and monocytes. Different T-cell populations are known to operate with opposite roles on the immune system; we did not make distinctions betweenT-cell subtypes so we cannot specify to what T-cell subpopulation Ang II upregulation was linked. However, in experimental models 8 weeks of exogenous Ang II treatment accelerated the development of atherosclerosis and prompted a switch from a stable to unstable plaque that was associated with a change in CD4+ T-lymphocyte activity; valsartan inhibited the effect of Ang II and significantly increased the frequency of Th2 and Treg cells.[26] In different atherosclerotic-prone models the proatherogenic properties of Th1 were shown and evidence from clinical investigations was collected that the upregulation of the Th1 immune response was overwhelming in patients with ACS,[27,28] and that an Ang II-due imbalance between Th1 and Th2 response might play a role in plaque rupture.[29,30] Recently, in healthy subjects, cultured Th1 and Th17 cells, as well as related cytokines including IFN-γ, were notably increased by the addition of Ang II in a dose-dependent manner, and the promoted effects were significantly inhibited after treatment with Ang II antagonists.[18] As a whole, these findings strongly suggest that the activation of T-cell RAS found in angina patients is part of the dysregulation in T-cell population underlying ACS.

Conclusions

Our findings strongly suggest that the activation of T-cell RAS found in unstable angina and not stable angina patients is part of the dysregulation in T-cell population function underlying ACS. T-cell RAS could play a major role in the promotion of the widespread coronary inflammation and plaque destabilisation in UA patients.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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