RESEARCH ARTICLE

Increased circulating RANTES in type 2 diabetes

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ABSTRACT. ***Aim:*** **The pro-atherogenic role of RANTES, a chemokine expressing pleiotropic activities, in the course of type 2 diabetes-related atherosclerosis has been well documented. However, it is not known which of the diabetes-related factors primarily influence serum RANTES levels in patients with type 2 diabetes. Our aim was to investigate relationships between several factors known to be related to an increased risk of atherosclerosis and serum RANTES levels in type 2 diabetic patients.** ***Methods:*** **A total of 168 subjects were examined, which included 138 patients with type 2 diabetes and 30 non-diabetic controls. Measurements of venous, fasting, plasma glucose, HbA_1c, lipid profile, 1,5-anhydro-D-glucitol (1,5-AG) plasma levels, homocysteine and the fasting, serum C-peptide levels were performed. Serum concentrations of RANTES were assayed using BD^{TM} Cytometric Bead Array tests. Peripheral insulin resistance was expressed according to a new index defined by Ohkura *et al*.** ***Results:*** **RANTES levels in type 2 diabetic patients correlated with 1,5-AG, fasting glycaemia, HbA_1c and the Ohkura index. Multivariate regression analysis was performed taking into consideration several factors related to the inflammatory process and atherosclerosis, namely the patient's age, diabetes duration, waist circumference, 1,5-AG, HbA_1c, lipid profile parameters, serum homocysteine levels and Ohkura index, as independent variables potentially influencing serum RANTES levels in type 2 diabetic patients. It is shown that RANTES concentrations in the serum is primarily dependent upon 1,5-AG plasma levels.** ***Conclusion:*** **Our results suggest that increased serum levels of RANTES in type 2 diabetic patients are closely related to postprandial (acute) hyperglycaemia.**

Key words: RANTES, type 2 diabetes, 1,5-anhydro-D-glucitol, postprandial hyperglycaemia

Over the past decade, our understanding of the importance of inflammation during all stages of atherosclerosis has greatly increased [1]. Moreover, a hypothesis has been proposed to explain the clinical course of type 2 diabetes that connects the disease to a state of subclinical, chronic inflammation [2]. Inflammation is accompanied by the emergence of numerous inflammatory biomarkers that comprise a vast array of substances, including cytokines such as the interleukins, acute phase proteins, adhesion molecules, interferons, chemokines, etc. [3].

Among the chemokines, CCL5/RANTES (CC chemokine ligand 5/regulated upon activation, normal T cell-expressed and secreted) is well known to express pleiotropic activities, especially a pro-inflammatory effect [4]. RANTES recruits leucocytes into sites of inflammation, increases neo-intima formation through monocyte recruitment, and plays an role in vascular repair through guidance of circulating mononuclear cells to the injury site and activation of resident vascular cells [5, 6]. Using C-reactive protein as a reference inflammatory biomarker, the authors describe the correlation between C-reactive protein, a well-established marker of inflammation, and serum RANTES levels [7]. Animal studies have highlighted the pro-atherogenic effects of both RANTES and its receptor CCR5. CCL5 is known to bind several receptors, including CCR1, CCR3, and CCR5: CCR1 and CCR5 are expressed on various cell types involved in atherosclerosis, e.g., monocytes/macrophages, T lymphocytes, or Th1-type cells, and specialise in mediating CCL5-triggered arrest and transendothelial diapedesis [8]. High levels of RANTES in plaque were found to be associated with an unstable plaque phenotype in humans [9].

Type 2 diabetes mellitus is characterized by crosstalk between many specific atherosclerosis- and inflammation–mediating factors. Systemic concentrations of RANTES are higher in individuals with type 2 diabetes than in control subjects [10]. It has been suggested that

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elevated RANTES levels in patients with type 2 diabetes may be a consequence of hyperglycaemia.

Chronic hyperglycaemia, such as that associated with diabetes, is well known to impair vascular function. However, recent evidence demonstrates the validity of postprandial hyperglycaemia as a cardiovascular risk factor [11, 12]. For several years 1,5-anhydro-D-glucitol (1,5-AG) has been suggested to be an indicator of metabolic control, being especially useful for detection of acute, short-term hyperglycaemic episodes [13, 14]. It was established that changes in plasma 1,5-AG levels reflect separate hyperglycaemic episodes appearing one-to-two days before the assay and postprandial hyperglycaemic excursions [15, 16]. Moreover, postprandial hyperglycaemiac spikes have been documented as triggering the inflammatory process [17]. Therefore the monitoring of plasma 1,5-anhydro-D-glucitol levels seems to be a useful method for every-day evaluation of the diabetes-induced inflammatory process.

The local role in adipose tissue may contribute to leukocyte infiltration and the proinflammatory state [18]. Up-regulation of RANTES and respective receptors in adipose tissue occurs in human obesity and is associated with increased systemic inflammation [19]; it might also play a part in mediating insulin resistance [4].

Homocysteine is known to take part in the development of atherosclerosis and vascular injury, and it has been suggested to contribute to the atherosclerotic process associated with diabetes mellitus [20, 21]. In addition, homocysteine directly increases the level of RANTES mRNA in isolated, normal human monocytes [22].

The role of RANTES in the course of type 2 diabetes - related atherosclerosis is well documented. However, it is not known which diabetes-related factors primarily influence serum RANTES levels in patients with type 2 diabetes. An understanding of this might provide interesting recommendations for therapeutic interventions.

Therefore, in this study we investigated relationships between factors related to the increased risk of atherosclerosis, and serum levels of the chemokine RANTES in type 2 diabetic patients.

MATERIALS AND METHODS

Patients

A total of 168 subjects were examined. They comprised 138 patients with type 2 diabetes and 30 non-diabetic controls. Patients with type 2 diabetes were managed for three months before examination, with one of the following therapeutic regimens: 82 individuals – with metformin alone (Glucophage XR® 500 at a dose of 500-1000 mg a day or Siofor 500® or Siofor 850®, 500-2550 mg daily); 25 subjects – with pre-mixed insulin analogues in combination with metformin (Novomix 30® or Novomix50® and Glucophage XR® 500 – 500-1000 mg a day or Siofor 500® or Siofor 850® - 500 – 2550 mg daily), and 31 patients – with pre-mixed insulin analogues only (Novomix 30® or Novomix50®).

Diabetes mellitus was diagnosed according to EASD and ADA criteria [23].

Each patient included in the study also received acetylsalicylic acid in antiplatelet doses, angiotensin-converting enzyme inhibitor or sartan, and a statin. Patients with underlying kidney or hepatic insufficiency, inflammatory or malignant disease, or with acute coronary episodes, were excluded from the study.

Approximately 50% of the type 2 diabetic patients suffered from stable angina – 66 subjects, and/or arterial hypertension – 70 individuals.

All patients were Caucasian. Patients were recruited from out-patient clinics in Poznan, Poland. Prior approval for all studies was given by the local Ethical Committee of the Poznan University of Medical Sciences, and all participants gave their informed consent.

Measurements

Measurements of venous, fasting, plasma glucose, the concentration of glycated hemoglobin HbA_{1c} in blood, total serum cholesterol levels, HDL and LDL fraction and serum triglyceride concentrations, 1,5-anhydro-D-glucitol plasma levels, fasting C-peptide serum levels and serum concentrations of CCL5 were performed. Blood samples, after overnight fasting and prior to drug administration, were taken from an antecubital vein and collected in EDTA-containing tubes. Tubes were placed on ice and centrifuged for 40 minutes, separated, and stored at -80°C prior to analysis.

Waist circumference was measured and BMI calculated for each patient. The evaluation of peripheral insulin resistance was not performed using HOMA index but expressed as novel index developed by Ohkura *et al*. [24], because a significant part of the population studied was treated with insulin.

CCL5 concentrations in serum were assayed with BD™ Cytometric Bead Array tests (Becton Dickinson), performed according to the manufacturer's instruction. Briefly, BD™ CBA Human Soluble Protein Flex Set consisting of a Human Soluble Protein Master Buffer Kit and Human Soluble Protein Flex Sets, which contain polymer beads coated with antibodies (capture beads). These beads are able to capture the studied analyte from suspension. Detection reagents constituted antibodies directed against the studied analytes. These antibodies were labeled with PE fluorochrome, with different fluorescence intensities for different analytes detected. This feature of the detection reagents allows assessment with a flow cytometer, because each detection reagent is strictly connected to a specified position on the matrix of the fluorescence channel. This allows for multiplexing of different flex sets and for evaluation of small amounts of studied material. Complexes of beads, proteins and antibodies labeled with PE were acquired with a FACS Canto flow cytometer (Becton Dickinson), and analysed with FCAP Array™ Software (Becton Dickinson).

The 1,5-anhydro-D-glucitol plasma level (1,5-AG), a marker of glucose excursions and postprandial hyperglycaemia [16], was measured using a modified column enzymatic method [16, 25]. Although the plasma level of 1,5-AG is not currently the standard marker for diabetes control, it is the only parameter which detects episodes of short-term hyperglycaemic. Recent data revealed the validity of acute, short-term hyperglycaemia as a cardiovascular risk factor [12], and for this reason the plasma 1,5-AG level was used instead of the routine markers. 1,5-AG, with its generally stable concentration in plasma of non-diabetic

persons, decreases rapidly (within one-to-two days) after a hyperglycaemic episode. 1,5-AG competes with glucose for transporting mechanisms in the renal tubules. Therefore, a decreased 1,5-AG level indicates retrospectively, a hyperglycaemic episode. The reference range for 1,5-AG is 13.8-30.2 mg/L [13, 16].

The C-peptide concentration in serum was measured using an ELISA with DIAsource Immuno Assay (C-peptide). C-reactive protein was analysed using an ELISA (R&D Systems).

Glucose concentrations and lipid parameters were measured using standard laboratory methods. HbA_1c [normal range: 4.1% (20 mmol/mol) – 6.0% (42 mmol/mol)] was assayed using an immunoturbidimetric method (COBAS Integra 400/700/800), standardised according to IFCC [26].

Statistical analysis

All results are expressed as mean ± SD and median. The Shapiro-Wilk test was used to assess the distribution of the variables examined. Statistical hypotheses were checked using the Mann-Whitney or Kruskall-Wallis test (in case of abnormal distribution) or with the t-test or ANOVA (in case of normal distribution). Regression analysis was used to account for the interaction between RANTES levels in serum and metabolic/anthropometric parameters. Multiple regression analysis was used to determine if 1,5-AG levels predict serum RANTES level, independent on other metabolic and anthropometric parameters.

All statistical analyses were performed using Statistica 8.0 (StatSoft). A p-value ≤ 0.05 was considered statistically significant.

Calculations

The Ohkura index was calculated as:

$$20/\left[\text{fasting C-peptide (nmol/L)} \times \text{fasting glucose (mmol/L)}\right]$$

RESULTS

Baseline characteristics of the groups investigated appear in *table 1*.

Subjects with type 2 diabetes and non-diabetic individuals were well matched for age, body mass index and waist circumference. Patients with type 2 diabetes exhibited significantly higher serum concentrations of RANTES than normoglycaemic individuals (*table 1B*).

The comparison of circulating serum RANTES levels between type 2 diabetic patients with stable angina and without coronary artery disease revealed higher levels in patients with stable angina – respectively 2654.6 ± 371.3 pg/mL *versus* 2353.9 ± 498.4 pg/mL.

By Spearman's analysis, we tested whether RANTES levels in type 2 diabetic patients correlated with key anthropometric and metabolic parameters, as well as with serum homocysteine levels. The strongest correlations were observed between serum RANTES levels and 1,5-AG, fasting glycaemia, HbA_1c and Ohkura index (*table 2*).

Table 1
The baseline characteristics of groups studied – part I

A				
	Type 2 diabetes n = 138 (68M/70F)		**Non-diabetic control n = 30 (12M/18F)**	
	x ± SD	**median**	**x ± SD**	**median**
Age (years)	61.5 ± 10.4	62.0	61.4 ± 6.4	60.0
Diabetes duration (years)	6.4 ± 5.1	5.0	-	
BMI (kg/m^2)	32.8 ± 7.5*	31.1	27.5 ± 4.8	28.8
Waist circumference (cm)	113.3 ± 0.5*	106.0	97.4 ± 14.4	95.0
Fasting glycaemia (mmol/L)	8.6 ± 2.3*	8.0	4.7 ± 0.5	4.9
HbA_{1c} (%)	7.3 ± 1.3*	7.1	5.8 ± 0.5	5.8
1,5 – Anhydro-D-glucitol (mg/L)	14.0 ± 6.2*	13.3	19.6 ± 5.8	18.8
Total cholesterol (mmol/L)	5.1 ± 1.2	5.1	4.8 ± 0.8	5.0
HDL-cholesterol (mmol/L)	1.3 ± 0.4*	1.2	1.6 ± 0.3	1.6
LDL-cholesterol (mmol/L)	2.9 ± 1.0	2.9	2.7 ± 0.7	2.7
Triglycerides (mmol/L)	2.0 ± 1.1*	1.7	1.3 ± 0.4	1.2
B				
	Type 2 diabetes n = 138 (68M/70F)		**Non-diabetic control n = 30 (12M/18F)**	
	x ± SD	**median**	**x ± SD**	**median**
C-peptide (nmol/L)	0.8 ± 0.5*	0.7	0.4 ± 0.1	0.4
Ohkura index	2.2 ± 0.4*	2.3	10.9 ± 2.0	10.9
Homocysteine (μmol/L)	14.8 ± 8.6*	11.7	9.0 ± 5.7	6.5
RANTES (pg/ml)	2497.7 ± 465.7*	2616.5	568.6 ± 65.5	598.9

* statistically significant against control group; P≤0.05

Table 2
Spearman correlation coefficients between serum RANTES levels and anthropometric and metabolic parameters.

n = 138	RANTES
Age	NS
Diabetes duration	NS
BMI	NS
Waist circumference	r = 0.25; p≤0.05
Fasting glycaemia	r = 0.46; p≤0.05
HbA_{1c}	r = 0.36; p≤0.05
1,5 – anhydro-D-glucitol	r = -0.55; p≤0.05
Total cholesterol	NS
HDL-cholesterol	NS
LDL-cholesterol	NS
Triglycerides	NS
C-peptide	r = -0.24; p≤0.05
Ohkura index	r = -0.45; p≤0.05
Homocysteine	r = 0.27; p≤0.05

NS- statistically non-significant.

Because the patient's age, diabetes duration, peripheral insulin resistance, hyperglycaemia, hyperlipidaemia and hyperhomocysteinaemia might be related to more advanced systemic inflammation, multivariate regression analysis was performed examining the patient's age, diabetes duration, waist circumference, 1,5-AG, HbA_1c, lipid profile parameters, serum homocysteine levels and Ohkura index as independent variables potentially influencing serum RANTES levels in type 2 diabetic patients. It was shown that the RANTES concentration in serum is primarily dependent upon postprandial hyperglycaemia expressed as the plasma 1,5-AG level (*figure 1*).

A comparison between type 2 diabetic patients that had varying chronic and/or postprandial hyperglycaemia was performed. It was revealed that satisfactory levels of HbA_1c are related to lower serum RANTES levels only in patients with 1,5-AG plasma levels, excluding postprandial (acute) hyperglycaemic episodes (*figure 2*).

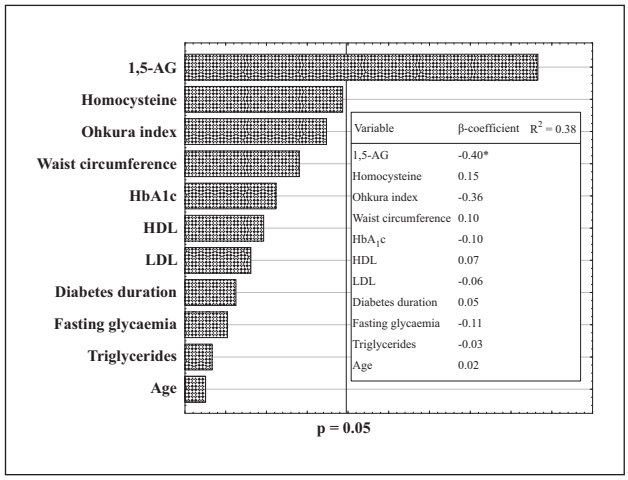

Figure 1
Multiple regression analysis, with serum RANTES level as the dependent variable.
* statistically significant for P≤0.05.

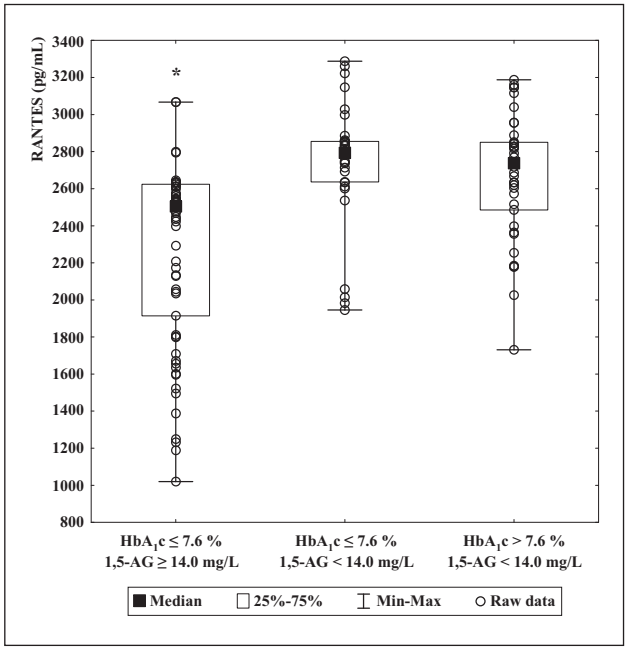

Figure 2
The comparison of serum RANTES levels among groups with varying in HbA_1c and/or 1,5-AG levels.
* statistically significant against other groups; P≤0.05.

DISCUSSION

Several lines of evidence indicate that RANTES plays a role in the pathogenesis of cardiovascular diseases. RANTES has emerged in recent years as a potential therapeutic target in the prevention of atherosclerosis [27, 28]. RANTES stimulates the adherence and transmigration of monocytes into the arterial wall [27] during the course of the atherosclerotic process. RANTES inhibition in animal models reduced atherosclerotic lesions [29]. Although this interpretation and the use of circulating RANTES level as a factor or even biomarker of progressive atherosclerosis remain debatable [9, 30], the relationship between serum RANTES levels and progression of coronary artery disease has been well described [7]. The authors found that serum RANTES levels correlated positively with total plaque burden and lipid-core volume. Therefore, they stated that circulating RANTES levels may help to identify the extent of atherosclerosis. Our study concerned a specific group of patients with an extremely high risk of progressive coronary artery disease i.e. patients with type 2 diabetes. Interestingly, although our patients suffered from only stable angina, it was found that serum RANTES levels were higher than in patients with coexisting coronary artery disease.

The relevance of activated immune cells and inflammation in the development of type 2 diabetes mellitus and atherosclerosis has recently been shown, and they appear to play a central role in mediating insulin resistance [31, 32]. A few studies have shown that RANTES is involved in adipose macrophage infiltration and the pathogenesis of insulin resistance. This chemokine is expressed by adipocytes in obesity-associated, chronic inflammation [33]. Furthermore, serum concentrations of RANTES were significantly elevated in obese *versus* lean subjects [19]. We found only a weak correlation between waist circumference and circulating RANTES and no correlation

between BMI and serum RANTES level. However, in our studies we confirmed the relationship between circulating RANTES and insulin resistance. The Ohkura index, calculated on the basis of fasting serum C-peptide and glucose levels, was used by us to estimate peripheral insulin resistance. Markedly lower Ohkura index values in type 2 diabetic patients from our study, in comparison to non-diabetic subjects, indicated clearly that the diabetic group was characterised by significantly increased peripheral insulin resistance coexisting with higher RANTES concentrations in the serum. Moreover, we found a correlation (r = -0.45) between the Ohkura index and circulating RANTES.

The inflammation during the course of type 2 diabetes is also known to be related to hyperlipidaemia [34]. Nevertheless, we found no relationship between circulating RANTES levels and lipid profile parameters in the group studied. Homocysteine has been described as the cholesterol of XXI century. To date, many cross-sectional, case-control, and cohort studies have linked hyperhomocysteinaemia with coronary artery disease [35]. Additionally, Sun *et al.* [22] revealed that RANTES is upregulated in monocytes from patients with hyperhomocysteinaemia. Our results report the potential, but relatively non-significant, effect of serum homocysteine levels on circulating RANTES.

Because it has been reported that hyperglycaemia might be a reason for increased serum RANTES levels [10], we focused our attention on the associations between fundamental hallmarks of diabetes; carbohydrate and lipid metabolism disturbances and circulating RANTES: intriguing correlations were revealed between this chemokine and fasting glycaemia (r = 0.46), HbA_1c (r = 0.36) and 1,5-AG, a marker of acute, postprandial hyperglycaemia (r = -0.55). The last correlation was especially interesting with respect to the results presented by Holmer *et al.* [36]. These authors reported that circulating concentrations of RANTES are profoundly affected in the postprandial period and are closely related to the increased serum RANTES levels. Many studies have reported a stronger association between cardiovascular risk and postprandial or post-load glucose than between cardiovascular risk and HbA1c [37-40]. Evidence is accumulating that postprandial hyperglycaemia is an independent risk factor for diabetes-associated complications and mortality, and plays a major role in activating oxidative stress, leading to endothelial dysfunction, one of the mechanisms responsible for vascular complications [41, 42]. Moreover, serum 1,5-AG levels were proposed as being useful in the identification of individuals at higher cardiovascular risk [43].

Taking into consideration all that has been mentioned above, we performed a multiple regression analysis including all the factors potentially modifying circulating RANTES: patient's age, diabetes duration, waist circumference, hyperglycaemia-related indices, lipid profile, homocysteine serum level, and index of insulin resistance as independent values. This analysis indicated that postprandial hyperglycaemia, independent of other factors, determines serum RANTES levels in type 2 diabetic patients and greater postprandial changes are related to higher levels of circulating RANTES.

To confirm that serum RANTES levels in the group studied are more closely related to postprandial glucose levels than to chronic hyperglycaemia expressed as HbA_1c levels, circulating serum RANTES levels were compared among three subgroups: 1) with satisfactory 1,5-AG and HbA_1c levels (1,5-AG$\geq$14.0 mg/L; $HbA_1c\leq$7.6 %); 2) with satisfactory HbA_1c and with 1,5-AG related to postprandial hyperglycaemic episodes (1,5-AG$<$14.0 mg/L; $HbA_1c\leq$7.6 %); 3) with unsatisfactory concentrations of both factors 1,5-AG and HbA_1c (1,5-AG $<$14.0 mg/L; $HbA_1c>$7.6 %). It is worth mentioning that serum RANTES levels were significantly higher in the groups with postprandial hyperglycaemia (groups 2 and 3), compared to group 1. Serum RANTES levels are much more obviously determined by postprandial hyperglycaemic episodes than by chronic hyperglycaemia.

CONCLUSIONS

Our results suggest that increased circulating RANTES in type 2 diabetic patients is closely related to postprandial (acute) hyperglycaemia.

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