Polymorphism A36G of the tumor necrosis factor receptor 1 gene is associated with PAI-1 levels in obese women

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Summary

The tumor necrosis factor (TNF) pathway may be implicated in etiopathogenesis of PAI-1 overexpression during obesity. The aim of this study was to investigate the influence of polymorphism A36G of the TNFRSF1A +36A/G on plasma concentrations of PAI-1 in 163 obese (31 with the metabolic syndrome, MetS) and 150 lean, healthy women. Genotypic and allele frequencies did not significantly differ between obese and lean subjects. TNFRSF1A genotypes were significantly associated with sTNFR1 plasma levels in obese women only (p<0.01); TNFRSF1A +36G/G obese carriers exhibited higher sTNFR1 and PAI-1 levels than A carriers (p<0.01 and p=0.05, respectively). In obese women, the presence of the MetS significantly potentiated the elevation of sTNFR1 and PAI-1 levels observed in the TNFRSF1A +36G/G carriers. Our results suggest that association between TNFRSF1A +36G/G genotype and the MetS renders obese women more prone to activation of the TNF pathway reflected by high circulating sTNFR1 and PAI-1 levels.

Keywords

Obesity, metabolic syndrome, inflammation, TNFR1, PAI-1

Introduction

Circulating PAI-1 is increased in obese subjects with the metabolic syndrome (MetS), and high PAI-1 levels may help to identify a high-risk population with the potential to induce both atherosclerotic disease and type 2 diabetes (1–3). Based on these results it has been hypothesized that PAI-1 could participate in the development of key features of the metabolic syndrome. This hypothesis has been sustained by the relationship described between PAI-1 gene polymorphisms, obesity and insulin resistance in population studies (4–6) and by experimental evidence linking PAI-1, insulin signaling (7–10) and weight gain (11–13).

Hence it is important to elucidate the mechanisms which lead to a rise in plasma PAI-1 levels during obesity. In the literature there has been some data on the involvement of potential mediators, one of which being tumor necrosis factor (TNF). Indeed, TNF has been involved in the mechanism of insulin resistance (14) and several clinical studies have observed an association between circulating TNF receptor 1 (TNFR1) levels and insulin-resistance indexes (15–18). Removal of both TNFRs in ob/ob mice led to significant reduction of PAI-1 mRNA in adipose tissue and PAI-1 plasma concentration. Administration of TNF neutralizing antibodies leads to an immediate decrease in plasma PAI-1 levels, proving a direct link between TNF and PAI-1 during obesity (19). In humans, the strong relationship observed between TNFRs and PAI-1 levels within adipose tissue (20) suggests a connection between the TNF pathway and PAI-1 during obesity.

TNFRSF1A gene codes for TNFR1. A silent substitution, TNFRSF1A +36 A/G, aids the capture of two main haplotypes, whose estimated frequencies were 0.42 and 0.26 in a French population (http://www.genecanvas.org). It was recently shown that TNFRSF1A +36 A/A genotype conferred a recessive protection against rheumatoid arthritis (21), a disease in which the TNF-TNFR system is highly activated. We hypothesized that
variations in the TNFRSF1A gene may be associated with PAI-1 levels during obesity. To our knowledge, no data associates this polymorphism with PAI-1 levels and the features of the metabolic syndrome. Therefore, the aim of our study was to examine the influence of the TNFRSF1A +36 A/G polymorphism on PAI-1 levels and on the inflammatory and metabolic disorders associated with obesity.

Methods

Subjects

One hundred sixty-three obese women (BMI ≥ 30 kg/m²) and 150 lean women were recruited consecutively from two outpatient clinics in Marseille: Centre d’Investigation Clinique and Centre de Dépistage et de Prévention de l’Athérosclérose. All the subjects were of caucasian origin. Four obese women were treated for diabetes mellitus, 24 for arterial hypertension and 12 for hyperlipidemia. None of the subjects had a history of cardiovascular events, liver disease, alcohol abuse and none reported any ongoing disease such as cancer or infection. In obese women, presence of the MetS was confirmed according to the World Health Organisation (WHO) criteria: fasting hyperinsulinemia (>18 I U/l or fasting glycemia >6.1 mM and at least two of the following: dyslipidemia (triglycerides ≥ 1.7 mM or HDL cholesterol < 1.0 mM); abdominal obesity (waist-to-hip ratio, WHR > 0.85) or BMI ≥ 30; hypertension ≥ 140/90 mmHg or antihypertensive treatment.

Blood samples were taken between 07:30 h and 09:00 h AM from fasting subjects, drawn into chilled trisodium-citrate tubes. Platelet-poor plasma was obtained after centrifugation for 30 min at 2000 g and 4°C, and stored at −80°C until analyzed. For DNA studies, venous blood was collected in EDTA tubes.

Informed written consent was obtained from all subjects. The study was conducted in accordance with the Declaration of Helsinki as revised in 2000. Our local institutional human research committee (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale de Marseille) approved the investigations (#9831, 04/28/1998).

Biochemical and immunological analyses

Blood lipids and glucose were determined by routine biochemical methods. Insulin was measured by the use of an ELISA (Insulin Kit, Dako, Denmark). Homeostasis model assessment (HOMA) was used to assess insulin resistance (insulin resistance = insulin (mIU/L) x glucose (mM) / 22.5) (22). Plasma interleukin-6 (IL-6) concentration was assayed by human Interleukin-6 UltraSensitive ELISA kit and plasma TNF concentration by human TNFα EASIA kit (both from Biosource Europe, Belgium). Specific ELISA was used to determine sTNFR1 antigen (R&D Systems, UK). C-reactive protein (CRP) was measured by an ultrasensitive immunonephelometry method (Dade Behring). PAI-1 antigen (ag) and activity (act) were assayed by Asserachrom PAI-1 (Diagnostica Stago, France) and Chromolize (BioPool International, Sweden), respectively.

DNA extraction and gene polymorphisms

Genomic DNA was prepared by standard salting-out techniques (23). For the determination of TNFRSF1A +36 A/G by PCR-
RFLP, PCR were carried out in a 25 µl reaction volume, using final concentrations of 200 nM for each primer, 200 µM for each dNTPs and 3.5 mM of MgCl₂, 75 ng of genomic DNA and 0.25 U of Taq DNA polymerase (Qbiotaq, Quantum, France).

Table 2: Genotypic and allele frequencies for TNFRSF1A polymorphism in obese and lean women. The number of subjects in each group is given in brackets. Differences between groups for genotype and allele were assessed using chi²-test with 2 and 1 df respectively.

<table>
<thead>
<tr>
<th>TNFRSF1A +36 A/G</th>
<th>Genotypes</th>
<th>p</th>
<th>Alleles</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA</td>
<td>AG</td>
<td>GG</td>
<td></td>
</tr>
<tr>
<td>Obese</td>
<td>0.33 (54)</td>
<td>0.49 (80)</td>
<td>0.18 (29)</td>
<td>0.58</td>
</tr>
<tr>
<td>Lean</td>
<td>0.40 (60)</td>
<td>0.46 (69)</td>
<td>0.14 (21)</td>
<td>0.41</td>
</tr>
</tbody>
</table>

The primers used were: F : 5’- GAGCCCAATGGGGGAGTGAGAG –3’ and R : 5’- ACCAGGCCGGGCGAGGAG –3’. Conditions were: first step of denaturation at 94°C during 1’30, followed by 40 cycles consisting in denaturation at 94°C, 30; annealing at 65°C, 45” and elongation at 72°C, 1’; and then a final step of 5’ at 72°C. Ten µl of the PCR product were digested by 2 U of MspAI I at 37°C during 90’, and the digested fragments were visualised by electrophoresis migration on 2% agarose gels stained with Ethidium Bromide (0.2 µg/ml final concentration). The expected sizes of the products after digestion were 183 bp for the A allele and 108 + 75 bp for the G allele.

Statistical analysis
Results were expressed as mean and standard deviation. Variables with a skewed distribution were log-transformed before analysis. Mean levels of continuous variables were compared between groups of women by ANOVA. When appropriate, one-way ANCOVA was used for comparisons of group means adjusted for confounders.

Differences between groups for genotype and allele frequencies and Hardy-Weinberg equilibrium were assessed using chi²-test. Association of TNFRSF1A +36 A/G gene polymorphism with plasma levels of the different variables studied was investigated by standard linear regression. The heterogeneity of the polymorphism effect in the two obese groups was tested by introducing the corresponding interaction term in the model. A p-value less than 0.05 was considered as statistically significant. Analysis was performed using Statistica software (StatSoft, Inc).

Results
Characteristics of obese and lean women are shown in Table 1. As expected, women with the MetS exhibited higher PAI-1 levels than women without the MetS. The exclusion of the four obese women with diabetes mellitus from the analyses did not affect the results (data not shown).

The TNFRSF1A +36 A/G polymorphism was in Hardy-Weinberg equilibrium. Genotypic and allele frequencies did not significantly differ between obese and lean subjects (Table 2), as well as between obese women with or without the MetS (data not shown).

Contribution of TNFRSF1A polymorphism to plasma concentrations of sTNFR1 was first studied. TNFRSF1A genotypes were significantly associated with sTNFR1 plasma levels in obese women only (AA: 785 ± 136, AG: 761 ± 160, GG: 880 ± 223 pg/ml, p<0.01, ANOVA) suggesting that this polymorphism could be of functional significance during obesity. The association between TNFRSF1A polymorphism and sTNFR1 levels...
was compatible with a recessive model ($p=0.43$), since the presence of the $G$ allele in a homozygous state was significantly associated with highest plasma levels of sTNFR1. Assuming this recessive model, lean women with the GG genotype exhibited the highest IL6 and lowest HDL-cholesterol levels. Obese women homozygous for the $G$ allele exhibited higher sTNFR1 and PAI-1 antigen concentrations than A carriers (Table 3). None of the other measured parameters followed this recessive model. These differences were observed despite the same BMI in the obese population ($BMI: GG: 33.7 \pm 3.9 \text{ vs. AA+AG: 33.6} \pm 3.5 \text{ kg/m}^2, p=0.85$).

Interestingly, the effect of the $TNFRSF1A +36G/G$ genotype on sTNFR1 and PAI-1 antigen levels was dependent on the presence of the MetS. Differences in sTNFR1 and PAI-1 antigen levels according to $TNFRSF1A +36A/G$ polymorphism were maintained only in the group of obese women with the MetS, but not in the group of obese women without MetS (Table 4). Obese women with the MetS and the $TNFRSF1A +36G/G$ genotype had the highest levels of sTNFR1 and PAI-1 (GG: $1047 \pm 141$ vs. AA+AG: $783 \pm 179 \text{ pg/ml}, p=0.002$ and GG: $122.7 \pm 77.0$ vs. AA+AG: $59.9 \pm 38.9 \text{ ng/ml}, p=0.02$; respectively). A significant interaction was found between the presence of the MetS and the $TNFRSF1A$ polymorphism on plasma sTNFR1 and PAI-1 levels ($p=0.02$). When looking at the percentage of obese MetS+ patients with the highest 5th percentile of sTNFR1 we found 0, 7 and 14% in the AA, AG and GG carriers, respectively. These percentages decreased to 0.8, 0 and 1.6% in the obese MetS- patients ($p=0.01$, chi²-test). Significance of the increased PAI-1 antigen concentration in obese women with the MetS carrying the G/G genotype disappeared after adjustment for sTNFR1 levels (ANCOVA, $p=0.22$).

### Discussion

The aim of this study was to test the hypothesis of an association between the $TNFRSF1A +36A/G$ polymorphism and plasma PAI-1 levels in obese women.

To our knowledge, the polymorphism in the first exon of $TNFRSF1A (+36 A/G)$ has not previously been studied in obese patients. In the present study, this polymorphism presents the same genotypic or allele frequencies between obese and lean women, suggesting that it contributes neither to the development of obesity nor to that of metabolic syndrome. The absence of a relationship between BMI, HOMA and the $TNFRSF1A +36 A/G$ polymorphism was not surprising: during genetic or nutritionally-induced obesity in mice, the deficiency in $TNFRSF1A$ gene never led to variations in fat accumulation (24), and a modest relationship has been observed between TNFR1 deficiency and insulin-sensitivity improvement.

Interestingly, lean subjects carrying the $TNFRSF1A +36 G/G$ genotype exhibited high IL6 and low HDL-cholesterol levels. Several data have already emphasized the relationship between lipoprotein concentrations and the TNF pathway in different pathologies, such as cardiovascular diseases (25) and rheumatoid arthritis (26). Usually, even in healthy populations, HDL cholesterol is inversely correlated with sTNFR1 plasma levels (27). The association observed between the $TNFRSF1A +36 A/G$ polymorphism and HDL-cholesterol levels reinforced the notion of the existence of a cause-effect relationship between the TNF pathway and lipids as previously mentioned (28, 29) and introduced a possible genetic control of this association. The link between $TNFRSF1A +36 G/G$ genotype and IL6 levels suggests that genetic variations in $TNFRSF1A$ may play a role in modulating the intensity of the inflammatory response in healthy, non-obese women. However, this genotype/phenotype association was not found with CRP. This is in line with previous results which showed that in patients with myocardial infarction, C-260T polymorphism of the CD14 gene was associated with plasma IL-6 but not with CRP or fibrinogen levels (30), suggesting a more direct link between the TNF pathway and IL6 than CRP. It has been speculated that polymorphisms within the TNFRs genes could alter either the binding of TNF or the receptor shedding, as found in $TNFRSF1A$ mutations associated with TNFR-associated periodic syndrome (TRAPS) (31), leading to a chronic inflammatory response.

Remarkably, these relationships did not remain in obese women, suggesting that obesity overcomes the effect of $TNFRSF1A +36G/G$ genotype on circulating IL6 levels. Obesity and especially the presence of a MetS revealed differences for sTNFR1 and PAI-1 levels according to the $TNFRSF1A +36A/G$ polymorphism. Obese women, carriers of the $TNFRSF1A +36G/G$ genotype, who completely fulfilled the WHO criteria for the MetS, exhibited the highest level of circulating sTNFR1 and PAI-1 levels. A significant statistical interaction was found between the $TNFRSF1A +36 G/G$ genotype and the MetS on sTNFR1 and PAI-1 levels. Thus, in obese women elevated circulating sTNFR1 and PAI-1 levels appeared to be the result of a gene (GG genotype) – environment (MetS) interaction. Conversely, in absence of MetS the effect of the $TNFRSF1A +36G/G$ on sTNFR1 and PAI-1 levels was absent.

### Table 4: Differences in plasma concentrations of sTNFR1 and PAI-1 in obese women with metabolic syndrome (MetS) and without it according to $TNFRSF1A +36 A/G$ polymorphism. $n$ represents the number of subjects. Data are mean ± SD (one-way ANOVA).

<table>
<thead>
<tr>
<th>$TNFRSF1A +36 A/G$ genotype</th>
<th>$n$</th>
<th>sTNFR1 (pg/ml)</th>
<th>PAI-1 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Obese women with MetS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>10</td>
<td>743 ± 112</td>
<td>63.6 ± 37.7</td>
</tr>
<tr>
<td>AG</td>
<td>13</td>
<td>815 ± 217</td>
<td>57.0 ± 41.1</td>
</tr>
<tr>
<td>GG</td>
<td>6</td>
<td>1047 ± 141</td>
<td>122.7 ± 77.0</td>
</tr>
<tr>
<td>p-value</td>
<td>0.007</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>p-value*</td>
<td>0.002</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td><strong>Obese women without MetS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>44</td>
<td>795 ± 141</td>
<td>40.9 ± 27.3</td>
</tr>
<tr>
<td>AG</td>
<td>67</td>
<td>751 ± 147</td>
<td>47.03 ± 38.6</td>
</tr>
<tr>
<td>GG</td>
<td>23</td>
<td>836 ± 222</td>
<td>50.5 ± 32.6</td>
</tr>
<tr>
<td>p-value</td>
<td>0.07</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>p-value*</td>
<td>0.07</td>
<td>0.3</td>
<td></td>
</tr>
</tbody>
</table>

*Tests were performed on log-transformed variables; ‘p-value using the validated recessive model (for comparison GG vs AA+AG).
Our results concur with a previous report that shows a relationship between this polymorphism and the severity of rheumatoid arthritis (21), and suggests either an association between the G allele and a chronic inflammatory response or a protective role of the A allele.

A direct regulatory role of this silent substitution on plasma protein levels is unlikely. More probable is that the linkage disequilibrium with a putative functional polymorphism gene could explain our finding. Recently, sequencing of the TNFRSF1A gene in 95 subjects with premature myocardial infarction showed that the TNFRSF1A +36A/G polymorphism was in strong linkage disequilibrium with two intronic fibronectin genes (C+186/in4T and G+10/in6A) and with one located in the promoter (G-609T) (32). The latter could be of functional importance as it is located in a putative response element for interferon when analyzed using MatInspector (http://www.genomaxit.de). In this case/control study a difference was found in the frequency of one TNFRSF1A polymorphism: carriers of the 92Q allele were twice as frequent in patients with myocardial infarction compared to controls, but in this study neither the TNFR1 levels were determined nor the presence of the metabolic syndrome.

In conclusion, we demonstrated that the TNFRSF1A +36A/G polymorphism influences plasma levels of sTNFR1 and PAI-1 in obese women. An interaction between the TNFRSF1A +36A/G polymorphism and the MetS was observed, suggesting a synergistic cooperation between the TNFRSF1A +36 G/G genotype and the MetS on systemic inflammation driven through the TNF pathway in obesity. Our results are limited by the small size of the sample. Larger studies are needed to confirm these results and to explain the mechanisms of the TNFRSF1A-MetS interaction.

Abbreviations

BMI, body mass index; TNF, tumor necrosis factor; PAI-1, plasminogen activator inhibitor type 1; MetS, metabolic syndrome; TNFRSF1A, TNF receptor superfamily 1A; sTNFR1, soluble TNF receptor 1; CRP, C-reactive protein; WHO, World Health Organization.

References