RANTES release by human adipose tissue in vivo and evidence for depot-specific differences

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Abstract

RANTES release by human adipose tissue was greater from the gastric fat pad compared with omental (P < 0.001) adipose tissue of lean and obese subjects and release of MCP-1 in subcutaneous and visceral adipose tissue, is frequently associated with adverse comorbidities. Furthermore, there are stronger links between adverse outcomes and the visceral adiposity compared with subcutaneous abdominal adipose tissue deposition (6, 17). To date, studies have looked at intra-abdominal visceral depots under the assumption of homogeneity. The recently described epicardial depot is also thought to behave in a similar way to visceral depots (19). However, differences in intrinsic properties of adipose tissue, its anatomic location and pattern of venous drainage, and/or genetic/environmental influences may contribute to make these depots more heterogeneous (16).

With this in mind we hypothesized that there are depot-specific differences in the production of RANTES from human adipose tissue, and in this study we aimed to assess the relative contributions of the various depots by looking at 1) in vivo release in lean and obese subjects across the abdominal subcutaneous depot; 2) ex vivo production from two different visceral adipose tissue depots, one in close proximity to the gastrointestinal tract (gastric fat pad) and the other further away (omental), as well as abdominal subcutaneous adipose tissue from morbidly obese subjects undergoing bariatric surgery; and 3) ex vivo production from the epicardial fat, in close proximity to the myocardium, and the thoracic subcutaneous adipose tissue from lean and obese subjects, free of coronary artery disease, who underwent cardiac surgery. In addition, two previously described proinflammatory cytokines, monocyte chemoattractant protein-1 (MCP-1) and interleukin-6 (IL-6), were used as reference.
Materials and Methods

Subjects

Study 1: arteriovenous differences of chemokines in lean and obese subjects. Healthy Caucasian volunteers were recruited by means of advertisement [age 41.0 (37.3–61.0) yr]. Lean subjects had body mass index (BMI) ≤27, and obese subjects had BMI ≥30 kg/m². Eleven lean (1 woman, 10 men) individuals [BMI 24.5 (23.0–26.3) kg/m²] and eight obese (4 women, 4 men) subjects [BMI 43.0 (34.8–45.8) kg/m²] were recruited. Arteriovenous differences of RANTES, MCP-1, and IL-6 were determined in the morning after an overnight fast.

Briefly, cannulas were inserted, under local anesthesia, into a radial artery and a superficial epigastric vein draining the abdominal subcutaneous adipose tissue (4, 14). Lines were kept patent by a slow infusion of isotonic saline. Blood samples were taken simultaneously from the two sites. Previous work has shown that venous blood from superficial epigastric veins approximates the effluent from the subcutaneous adipose tissue bed, and arteriovenous differences across abdominal adipose tissue yield results in good agreement with those of microdialysis studies (21).

Study 2(a): chemokine release from abdominal subcutaneous and visceral deposits. Abdominal subcutaneous, omental, and gastric fat pad biopsies were obtained from obese [BMI 43.1 (36.9–47.6) kg/m²] Caucasian patients (n = 14, all women) undergoing laparoscopic bariatric surgery [age 44.5 (42.8–48.5) yr]. Recruitment was from the preoperative clinic.

Study 2(b): chemokine release from epicardial and thoracic subcutaneous deposits. Thoracic subcutaneous and epicardial adipose tissue samples were obtained from an additional group of Caucasian patients [age 65.5 (60.5–73.8) yr] undergoing cardiac surgery because of valvular heart disease. There were eight lean (7 women, 1 man) subjects [BMI 24.7 (22.3–25.4) kg/m²] and eight obese (3 women, 5 men) individuals [BMI 31.6 (29.2–37.1) kg/m²]. These patients were also recruited from the clinic. Patients with diabetes, coronary artery disease, malignancy or terminal illness, connective tissue disease or other inflammatory conditions likely to affect cytokine levels, or severe uncontrolled hypertension, immunocompromised subjects, and subjects with substance abuse or other causes for poor compliance were excluded from both studies 2(a) and 2(b). Blood samples, after an overnight fast, were taken from an antecubital vein on the day of the operation between 7:30 and 9:30 AM, separated, and stored at −80°C until analysis.

Local Ethics Committees approved the studies, and written informed consent was obtained from all participants.

Anthropometric Measurements

BMI was calculated as the weight (kg) divided by the square of the height (m²). Blood pressure was measured with a random zero sphygmomanometer (Hawksley Gelman, Lancing, UK).

Organ Cultures

Adipose tissue samples (0.2 g of abdominal subcutaneous, omental, and gastric fat pad; 0.05 g of thoracic subcutaneous and epidermal adipose tissue) were finely minced and incubated for 24 h in serum-free medium (Cellgro, Mediatech) containing 1% (vol/vol) penicillin-streptomycin (GIBCO, Paisley, UK) at 37°C in 5% CO₂. Supernatant was harvested, snap-frozen in liquid N₂, and stored at −80°C until analysis.

Cytokine Protein Array

The assay was carried out per manufacturer’s instructions (Proteome Profiler Array, Human Cytokine Array Panel A, R & D Systems) with adipose tissue conditioned media from five patients (2 bariatric and 3 cardiac surgery). Capture antibodies to 36 cytokines/chemokines were spotted in duplicate on nitrocellulose membranes. Membranes were exposed to X-ray film (Amersham Hyperfilm ECL, GE Healthcare) for up to 3 min. Films were scanned and quantified as pixel density with Adobe Photoshop. Signals from the negative control spots (background value) were subtracted from each spot. Positive control spots were taken as 100% and other spots shown relative to this. Spots with densities <10% of the positive controls were considered negative.

Adipose Tissue Fractionation

Briefly, 0.5 g of abdominal subcutaneous and omental adipose tissue from a subset of patients was cleaned from vessels, cut into small pieces, and digested with 0.2% collagenase in a shaking water bath at 37°C and 100 rpm for 1 h. The stromal cell pellet was separated from mature adipocytes by centrifuging at 2,060 relative centrifugal force (rcf) for 15 min at 4°C. The adipocyte and stromovascular (SV) fractions were stored at −80°C until analysis.

RNA Extraction, cDNA Synthesis, and PCR

Gastric fat pad and abdominal subcutaneous and omental adipose tissue (0.2 g) and thoracic and epicardial adipose tissue (0.1 g) were ground in liquid N₂. RNA was extracted with a commercial kit (RNeasy, Qiagen, Crawley, UK). The concentration and purity of isolated RNA were assessed by measuring the optical density at 260 nm (OD₂₆₀) and 280 nm (OD₂₈₀). cDNA was synthesized with reverse transcriptase and random oligonucleotide primers (Applied Biosystems, Roche). Specific primer sequences for Taqman RT-PCR analysis were designed with Express (Applied Biosystems, Roche). β-Actin was used as housekeeper gene. Samples were analyzed in triplicate.

Assays

Plasma glucose concentration was assayed with glucose oxidase reagent (Beckman). Serum triglycerides and total cholesterol were

Table 1. In vivo release of chemokines by abdominal subcutaneous adipose tissue of lean and obese subjects

<table>
<thead>
<tr>
<th>Variables</th>
<th>Lean (n = 11; 10 M, 1 F)</th>
<th>Obese (n = 8; 4 M, 4F)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI, kg/m²</td>
<td>24.5 (23.0–26.3)</td>
<td>43.0 (34.8–45.8)</td>
<td>0.001</td>
</tr>
<tr>
<td>Arterial MCP-1, pg/ml</td>
<td>104.5 (63.6–125.6)</td>
<td>91.8 (89.7–93.9)</td>
<td>0.7</td>
</tr>
<tr>
<td>Venous MCP-1, pg/ml</td>
<td>108.0 (84.3–149.2)</td>
<td>123.0 (84.0–164.2)</td>
<td>0.6</td>
</tr>
<tr>
<td>V-A difference of MCP-1, pg/ml</td>
<td>0.0 (–5.6–19.3)</td>
<td>29.2 (3.6–56.4)</td>
<td>0.2</td>
</tr>
<tr>
<td>Arterial RANTES, ng/ml</td>
<td>7.4 (4.1–20.5)</td>
<td>16.2 (9.1–31.0)</td>
<td>0.2</td>
</tr>
<tr>
<td>Venous RANTES, ng/ml</td>
<td>12.9 (5.2–56.7)</td>
<td>31.6 (28.2–65.0)</td>
<td>0.2</td>
</tr>
<tr>
<td>V-A difference of RANTES, ng/ml</td>
<td>4.7 (–0.4–12.7)</td>
<td>14.6 (–13.2–28.4)</td>
<td>0.4</td>
</tr>
<tr>
<td>Arterial IL-6, pg/ml</td>
<td>0.9 (0.5–1.8)</td>
<td>6.3 (2.2–11.7)</td>
<td>0.001</td>
</tr>
<tr>
<td>Venous IL-6, pg/ml</td>
<td>1.4 (1.2–9.3)</td>
<td>41.2 (20.7–46.0)</td>
<td>0.001</td>
</tr>
<tr>
<td>V-A difference of IL-6, pg/ml</td>
<td>0.8 (0.3–7.2)</td>
<td>32.6 (17.1–36.7)</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Data are shown as medians [interquartile range (IQR)] for n subjects. Concentrations of chemokines in samples obtained simultaneously from the radial artery and the abdominal superficial epigastric vein draining the subcutaneous adipose tissue are shown. M, male; F, female; BMI, body mass index; MCP-1, monocyte chemotactic protein-1; RANTES, regulated on activation, normal T cell expressed and secreted; IL-6, interleukin-6; V-A difference, difference between concentrations measured in venous vs. arterial effluent. All comparisons between lean and obese subjects were done with Wilcoxon test for related samples.
assayed with commercial reagents (total cholesterol, Boehringer Mannheim, Lewes, UK; triglycerides, Roche Diagnostics, Welwyn Garden City, UK). HDL-cholesterol was measured by the same method after the low-density lipoproteins were quantitatively precipitated out by the addition of phosphotungstic acid in the presence of magnesium ions. LDL-cholesterol was calculated with the Friedewald formula (3). C-reactive protein (CRP) was assayed by a latex-enhanced immunoturbidimetric (agglutination) procedure, measuring the light scattered by anti-CRP antibody-latex complex. The turbidity was measured photometrically at 340 nm, which related to the concentration of the antigen (CRP). All lipid and CRP assays were performed by Dr. David Wickens, Chemical Pathology, Whittington Hospital (London, UK). RANTES, MCP-1, and IL-6 were measured with human two-site ELISAs (R & D Systems, Abingdon, UK). Human serum IL-6 concentrations were assayed with the high-sensitivity ELISA, with a limit of detection of 0.09 pg/ml. All other ELISAs for the measurements of chemokine levels in culture supernatants or serum were of normal sensitivity with inter- and intra-assay coefficients of variation <10% (R & D Systems).

**Statistical Analysis**

Data were analyzed with SPSS version 14 for Windows software (Statistical Package for the Social Sciences, SPSS UK, Chertsey, UK). Normality of distributions was tested with the Kolmogorov-Smirnov test. Cytokine concentrations are shown as median [interquartile range (IQR)] in text and in Tables 1 and 2. Comparison of lean and obese subjects was by Mann-Whitney U-test. Arteriovenous and between-depot differences were analyzed by Wilcoxon test. Spearman rank correlations were used for the bivariate analysis. Significance was defined as \( P \leq 0.05 \).

**RESULTS**

**Study 1: Arteriovenous Differences of Chemokines in Lean and Obese Subjects**

Chemokine concentrations are shown in Table 1. In lean subjects (\( n = 11 \)), there was significant release of RANTES (\( P = 0.04 \)) and IL-6 (\( P = 0.003 \)), but not MCP-1 (\( P = 0.33 \)), from
the abdominal subcutaneous adipose tissue (Table 1, Fig. 1). However, in obese subjects ($n = 8$), there was significant release of all three molecules by the subcutaneous adipose tissue: RAN-
TES ($P = 0.04$), IL-6 ($P = 0.01$) and MCP-1 ($P = 0.04$) (Table 1, Fig. 1).

IL-6 levels were significantly elevated both locally and systemically in obese subjects compared with lean individuals (Table 1). Accordingly, local IL-6 secretion by the subcutaneous depot, assessed as arteriovenous difference, was enhanced in the obese subjects ($P = 0.003$). Obesity did not further affect RANTES and/or MCP-1 release from this depot.

Arterial and venous concentrations of RANTES showed significant interaction ($r = 0.70, P = 0.007$). Arteriovenous differences of RANTES correlated positively and significantly with arteriovenous differences of IL-6 ($r = 0.48, P = 0.04$) and MCP-1 ($r = 0.47, P = 0.04$).

**Study 2**

CRP, as an index of inflammation, was not different between the lean, obese, and morbidly obese patients in studies 2(a) and (b). Therefore, correlations were sought in the whole group. Serum CRP correlated significantly with BMI ($r = 0.37, P = 0.05$) and fasting serum insulin ($r = 0.44, P = 0.04$). No significant associations with serum lipids, glucose, or IL-6 were apparent. However, CRP correlated significantly with serum RANTES ($r = 0.45, P = 0.02$) but not with levels secreted from the different depots.

Cytokine protein array data from the patients studied ($n = 5$: 2 bariatric and 3 cardiac surgery) showed that of the 36 molecules specified in the array, 17 (CD40 ligand, granulocyte macrophage colony-stimulating factor (GM-CSF), IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12 p70, IL-17, IFN-inducible protein-10 (IP-10), I-TAC, macrophage inflammatory protein (MIP)-1α and -β, stromal cell-derived factor (SDF)-1, tumor necrosis factor (TNF)-α, soluble triggering receptor expressed

Fig. 2. Adipose tissue depot-specific differences in the release of cytokines in obese subjects by protein array. With conditioned media from adipose tissue from bariatric ($n = 2$) and cardiac ($n = 3$) surgery patients, $19$ of a possible 36 cytokines were detectable in human adipose tissue culture supernatants. Growth-related protein α (GROα), IL-6, IL-8, macrophage migration inhibitory factor (MIF), and type 1 plasminogen activator inhibitor (PAI)-1 had densities above 50% of the positive control. Positive control spots were assigned a value of 100%, and other spots are shown relative to this. Spots with densities <10% of the positive control were considered negative. RANTES release from gastric fat pad (GFP) was highest, compared with that from the other depots. Histogram shows mean optical density of the spots expressed as % of positive control of the results from the 5 patients; representative blots obtained from 1 bariatric and 1 cardiac surgery patient are shown at bottom. PC, positive control; Om, omental; SC, subcutaneous; Epi, epicardial; G-CSF, granulocyte colony-stimulating factor; sICAM-1, soluble ICAM-1; IL-1RA, IL-1 receptor antagonist.
significant correlations between the release of RANTES from the gastric fat pad and the other chemokines.

mRNA EXPRESSION. RANTES mRNA, in agreement with protein release data, was significantly higher in the gastric fat pad samples, compared with that from the abdominal subcutaneous or omental adipose tissues. Both the SV and adipocyte fractions expressed detectable levels of RANTES and adiponectin mRNA, with RANTES higher in the SV fraction and adiponectin mainly in adipocytes.

Study 2(b): chemokine release from epicardial and thoracic subcutaneous depots. The patients undergoing cardiac surgery were dichotomized into lean (n = 8; BMI ≤ 27 kg/m²) and obese (n = 8; BMI > 27 kg/m²) subjects (Table 2). While in the lean group epicardial adipose tissue released significantly less RANTES compared with thoracic subcutaneous tissue (P = 0.04), this was not apparent in obese subjects (P = 0.31). Epicardial RANTES release was greater in the obese subjects (P = 0.04), whereas the subcutaneous release of RANTES showed greater variability and did not differ between the groups (Fig. 3). The data from the protein array experiment were in agreement for the lean subject; however, RANTES was poorly detected by this technique in the obese subject studied (Fig. 2).

Epicardial RANTES release correlated positively and significantly with systemic RANTES levels (r = 0.59, P = 0.03) and with markers of metabolic dysfunction: BMI (r = 0.60, P = 0.02), triglycerides (r = 0.61, P = 0.03), and diastolic blood pressure (r = 0.61, P = 0.02). This was not noted for any of the other depots studied.

Systemic levels of RANTES, MCP-1, and IL-6 were higher in the two obese groups compared with the lean subjects (see Table 2). When all subjects were assessed collectively, no male (n = 20)-female (n = 29) difference was seen in serum RANTES levels, in either the normal-weight (P = 0.22) or obese (P = 0.10) group. Age was not significantly associated with systemic cytokines (age and RANTES r = −0.15, P = 0.35; IL-6 r = −0.18, P = 0.35; MCP-1 r = 0.19, P = 0.25).

DISCUSSION

In this study, we demonstrate release of RANTES by the abdominal subcutaneous adipose tissue depot in vivo, in both lean and obese individuals. We also report mRNA expression and ex vivo secretion of RANTES, by protein array and ELISA, from two subcutaneous (abdominal and thoracic), two visceral (omental and gastric fat pad), and epicardial adipose tissue depots. Wu et al. (24) recently reported expression of RANTES and of its associated receptor CCR5 in human subcutaneous and visceral (perigastric omental) adipose tissue. Another recent study reported higher expression of CD68 and RANTES in both subcutaneous and omental adipose tissue from obese subjects, and, furthermore, the expression of RANTES and its receptors correlated positively with CD68 expression (8). However, our study is the first to describe RANTES protein

Table 2. Anthropometric and metabolic characteristics of surgical patients

<table>
<thead>
<tr>
<th>Variables</th>
<th>Study 2b (cardiac surgery)</th>
<th>Study 2a (bariatric surgery)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lean (n = 8; 1 M, 7 F)</td>
<td>Obese (n = 8; 5 M, 3 F)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>24.1 (1.6)</td>
<td>32.8 (4.4)†</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>138.1 (24.5)</td>
<td>147.5 (18.5)</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>68.8 (9.2)</td>
<td>75.6 (15.2)</td>
</tr>
<tr>
<td>Total cholesterol, mmol/l</td>
<td>5.2 (1.6)</td>
<td>5.1 (1.2)</td>
</tr>
<tr>
<td>HDL-cholesterol, mmol/l</td>
<td>1.8 (0.5)</td>
<td>1.3 (0.3)</td>
</tr>
<tr>
<td>LDL-cholesterol, mmol/l</td>
<td>2.9 (1.2)</td>
<td>2.7 (1.3)</td>
</tr>
<tr>
<td>Triglycerides, mmol/l</td>
<td>0.9 (0.4)</td>
<td>2.2 (0.6)†</td>
</tr>
<tr>
<td>Glucose, mmol/l</td>
<td>5.1 (0.5)</td>
<td>5.9 (1.7)</td>
</tr>
<tr>
<td>Insulin, pmol/l</td>
<td>24.1 (23.9–31.5)</td>
<td>25.8 (23.8–31.8)</td>
</tr>
<tr>
<td>Adiponectin, μg/ml</td>
<td>15.3 (9.1–20.3)</td>
<td>10.2 (9.6–12.4)</td>
</tr>
<tr>
<td>Leptin, ng/ml</td>
<td>9.8 (6.9–19.2)</td>
<td>11.0 (6.7–46.9)</td>
</tr>
<tr>
<td>IL-6, pg/ml</td>
<td>1.5 (1.4–2.8)</td>
<td>2.5 (1.7–5.5)</td>
</tr>
<tr>
<td>MCP-1, pg/ml</td>
<td>110.2 (101.6–210.0)</td>
<td>275.9 (192.5–379.0)*</td>
</tr>
<tr>
<td>RANTES, ng/ml</td>
<td>14.5 (3.3–46.4)</td>
<td>29.4 (15.1–72.0)</td>
</tr>
</tbody>
</table>

Data are shown as means (SD) or medians (IQR) for n subjects. Obese and morbidly obese groups were compared with the lean group by Mann-Whitney test.

*Difference significant at 0.05 level (2-tailed); †difference significant at 0.01 level (2-tailed).
release from various human adipose tissue depots rather than its expression, with some insight into its potential to act as a paracrine or endocrine factor.

Adipose tissue is known to express a constantly increasing number of chemokines and cytokines, and our protein array data confirm these findings. However, not all of these are actively secreted into the circulation (14). We show that systemic levels of RANTES are ~100-fold higher than those being released from any of the adipose tissue depots studied. Therefore, it would be reasonable to suggest that while adipose tissue is able to produce this chemokine, its contribution to systemic levels may be less significant. This was further confirmed by a lack of association between its circulating levels and indexes of obesity. In contrast, IL-6 production from the subcutaneous (14) as well as the visceral (5) depots contributes significantly to the systemic circulation, with the potential to have endocrine effects on various organs, especially in obesity.

Wu et al. (24) found higher RANTES mRNA levels in visceral compared with subcutaneous adipose tissue in obese humans. In our study we assessed two distinct intra-abdominal depots, as well as subcutaneous adipose tissue, simultaneously from the same morbidly obese patients undergoing bariatric surgery. The gastric fat pad is in close proximity to the stomach, sitting in the angle of His. It is not described in the literature in detail, and we are not aware of studies comparing it to the omental depot. This depot is highly vascularized and may become thickened in morbidly obese subjects, while its resection may prevent obstruction after gastric banding (18, 20). The gastric fat pad releases significantly more RANTES than either the omental or subcutaneous depots, perhaps because of local regulation by gastrointestinal factors. The close association between omental and subcutaneous RANTES, but not gastric fat pad RANTES, may also imply that this depot is independently regulated.

The epicardial adipose tissue is in close proximity to the myocardium and the coronary arterial tree (10). Its increased ability for fatty acid incorporation and lipogenesis on one hand, and for fatty acid release on the other, implies that it primarily serves as a local energy source to the adjacent myocardium and/or as a scavenger of circulating free fatty acids, which are known to be toxic for cardiomyocytes and can also affect electrical conductivity in the heart (10, 11). Previously, in a population with coronary artery disease, increased expression of IL-1β, TNF-α, IL-6, IL-6 soluble receptor, and MCP-1 was described in epicardial compared with subcutaneous adipose tissue (13). Here, we report ex vivo release of RANTES from the epicardial adipose tissue in individuals free of coronary artery disease. Epicardial, but not subcutaneous, RANTES release correlates positively with BMI and is enhanced in obese individuals.

In summary, we show RANTES production by human subcutaneous adipose tissue in vivo and release of this chemokine from novel depots, the gastric fat pad and epicardial fat. While epicardial RANTES is related to obesity, neither systemic RANTES nor its release from the subcutaneous and the abdominal visceral adipose tissue is a good marker of adiposity. There is significant heterogeneity in the release of RANTES from the various depots, a finding that suggests differential regulation by autocrine/paracrine factors. A weakness of this study is that the abdominal and thoracic depots are from different subjects, and therefore direct comparisons are difficult. Elevated RANTES expression in the adipose tissue of diet-induced obese male mice is associated with increased T-cell infiltration, suggesting paracrine chemotactic effects (24). Given that in other cell populations RANTES affects intracellular pathways that are central to adipocyte biology (25, 26), it would be interesting in the future to evaluate adipose tissue as a target, rather than as a source, of RANTES.

GRANTS

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