CLINICAL STUDY

Expression of tumor necrosis factor (TNF)- α protein in the subcutaneous and visceral adipose tissue in correlation with adipocyte cell volume, serum TNF- α , soluble serum TNF-receptor-2 concentrations and C-peptide level

Gábor Winkler¹, Sándor Kiss², László Keszthelyi², Zoltán Sági³, Iván Öry¹, Ferenc Salamon¹, Margit Kovács⁵, Péter Vargha⁶, Orsolya Szekeres¹, Gábor Speer⁴, István Karádi⁵, Márta Sikter⁷, Edit Kaszás⁷, Otto Dworak⁹, Gábor Gerő⁸ and Károly Cseh⁷

Departments of ¹Internal Medicine II, ²Surgery and ³Pathology, St John's Hospital ⁴I and ⁵III Clinic of Internal Medicine, ⁶Biometric Research Group, Semmelweis University, Departments of ⁷Internal Medicine I and ⁸Pathology, Károlyi Hospital, Budapest, Hungary and ⁹Institut für Pathology, Fürth, Germany

(Correspondence should be addressed to G Winkler; Email: winkler.gabor@axelero.hu)

Abstract

Objective: The aim of the study was to evaluate the expression of tumor necrosis factor (TNF)- α protein in the subcutaneous and visceral adipose tissue in correlation with adipocyte cell volume, serum TNF- α , soluble TNF-receptor-2 (sTNFR-2) and indirect parameters of insulin resistance in overweight/obese and lean healthy persons.

Design: A cross-sectional case-control study was used.

Patients: Twenty-eight overweight/obese probands with normal glucose tolerance (BMI > 27 kg/m²) and 15 lean people (BMI < 25 kg/m²), all of them undergoing planned surgical operation, participated in the study.

Methods: Two to four grams of subcutaneous and visceral adipose tissue were removed and studied using semi-quantitative immunohistochemical staining of the TNF- α protein. Serum TNF- α , sTNFR-2 (ELISA) and fasting C-peptide (RIA) were measured.

Results: TNF- α protein was expressed in adipocytes of both depots. The expression was evaluated visually and found to be greater in the obese patients. Significantly higher serum TNF- α (5.58 ± 0.87 pg/ml vs 4.21 ± 0.55 , mean $\pm$ s.d., $P < 0.01$, Mann–Whitney) and sTNFR-2 levels (7.84 ± 3.56 ng/ml vs 4.59 ± 1.35 , $P = 0.005$) were found in the obese subgroup in correlation with the fasting C-peptide level ($r = 0.49$, $P = 0.003$; and $r = 0.74$, $P = 0.001$) and the C-peptide/blood glucose ratio ($r = 0.47$, Spearman, $P = 0.005$; and $r = 0.70$, $P = 0.001$). The cell volume of both adipocyte depots was found to have a significant positive correlation with serum TNF- α and sTNFR-2 levels in the total group of patients (subcutaneous: $r = 0.52$, $P = 0.0003$; $r = 0.69$, $P < 0.0001$; visceral: $r = 0.65$, $P < 0.0001$; $r = 0.63$, $P < 0.0001$) and in both subgroups.

Conclusions: Adipocyte cell volume of both the subcutaneous and visceral fat depots may be determinants of TNF- α , sTNFR-2 production and obesity-linked insulin resistance.

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Introduction

Tumor necrosis factor (TNF)- α has been reported to be upregulated in the fat tissue of different animal models of obesity and also in humans. The increased expression of the cytokine has also been found to be associated with insulin resistance (1–4). Depot-specific differences in the production of adipocytokines – e.g. leptin in the subcutaneous, interleukin-6 in the omental adipose tissue, etc. – have also been observed (5). Conflicting results concerning the differences

in subcutaneous and visceral expression of TNF- α have been published (2, 6, 7).

The receptors of TNF- α (TNFR-1 and TNFR-2) are involved in the regulation of insulin signaling and insulin resistance in adipocytes (8, 9), TNFR-2 has also been observed to increase the differentiation of pre-adipocytes, and the concentration of soluble TNFR-2 (sTNFR-2) was found to be more elevated in obese patients than the concentration of sTNFR-1 (1).

Adipocyte size may be an important determinant of cytokine synthesis. For example, larger fat cells contain

more leptin than smaller adipocytes obtained from the same individual (10). In an animal model, the expression of TNF- α in adipocytes also correlated with cell size (11).

In the light of these observations, the aim of the present study was to evaluate the expression of TNF- α protein in surgically removed subcutaneous and visceral adipose tissue of overweight and lean patients, and to analyze its correlation with adipocyte cell volume, serum TNF- α and sTNFR-2 concentrations, and indirect parameters of insulin resistance (fasting and post-challenge C-peptide levels and C-peptide/blood glucose ratios).

Subjects and methods

After having their written informed consent approved by the ethical committee of St John's Hospital, Budapest, 43 patients with normal glucose tolerance and without known underlying metabolic, autoimmune, infectious or malignant disorders were involved in the study. Subsequently, participants were divided into two subgroups according to their BMI: overweight/obese (13 males, 15 females, BMI $> 27 \text{ kg/m}^2$) and lean persons (seven males, eight females, BMI $< 25 \text{ kg/m}^2$). All patients and controls underwent a planned surgical intervention (cholecystectomy or herniotomy). Prior to the intervention, a 75 g oral glucose tolerance test (OGTT) combined with fasting and post-challenge (120 min) C-peptide

determinations, fasting serum TNF- α , and sTNFR-2 measurements were carried out. Clinical, anthropometric and laboratory parameters of the participants are summarized in Table 1. With the exception of the 120 min OGTT C-peptide and blood glucose values, all laboratory parameters were determined in the fasted state.

During the surgical intervention, 2–4 g of subcutaneous and omental fat tissue obtained from similar anatomic regions were removed by scalpel from each proband, and immediately formalin fixed and then paraffin embedded for immunohistochemical determination of TNF- α . Sections were incubated with antihuman mouse monoclonal-TNF- α (Cambio, Cambridge, UK) at 37 °C for 30 min, at a working dilution of 1:200. Subsequently, biotinylated multilink antibodies (Novocastra, Newcastle, UK) were applied for 15 min at a dilution of 1:15 and streptavidin peroxidase was added for 15 min at a dilution of 1:15 using a previously described procedure (12). The reaction was developed with amino-ethyl-carbasol chromogen substrate (red-dish color), and for the visual evaluation a Mayer hematoxylin staining was also carried out for 20 min. The staining was graded microscopically ($\times 200$), semi-quantitatively as follows: no staining (grade 0), weak (grade 1), medium (grade 2) and strong reaction (grade 3). The expression of TNF- α was scored according to the grade (0, 1, 2 or 3), the individual scores were summarized and the total score for the overweight/obese and the lean subgroups were calculated

Table 1 Clinical, anthropometric and laboratory parameters of the overweight/obese and lean subgroups (means $\pm$ S.D.).

Clinical, anthropometric and laboratory parameters	Subgroups	
	Overweight/obese subjects	Lean probands
Number of cases (men/women)	28 (13/15)	15 (7/8)
Age (years)	46.45 $\pm$ 13.48	43.60 $\pm$ 12.65
BMI (kg/m^2)	29.56 $\pm$ 2.24	23.15 $\pm$ 1.80**
Serum TNF- α (pg/ml)	5.58 $\pm$ 0.87	4.21 $\pm$ 0.55**
Serum sTNFR-2 (ng/ml)	7.84 $\pm$ 3.56	4.59 $\pm$ 1.35*
Serum fasting C-peptide (ng/ml)	2.91 $\pm$ 1.20	1.43 $\pm$ 0.58**
Serum 120 min C-peptide (ng/ml) ^a	10.02 $\pm$ 3.73	1.98 $\pm$ 0.68**
Fasting blood glucose (mmol/l)	5.54 $\pm$ 0.79	5.13 $\pm$ 0.63
120 min blood glucose (mmol/l) ^a	7.37 $\pm$ 1.80	6.77 $\pm$ 0.90
C-peptide/blood glucose ratio, fasting	0.53 $\pm$ 0.21	0.28 $\pm$ 0.10**
C-peptide/blood glucose ratio, 120 min ^a	1.39 $\pm$ 0.52	0.29 $\pm$ 0.10**
HbA _{1c} (%)	5.70 $\pm$ 0.65	5.33 $\pm$ 0.64
Serum cholesterol (mmol/l)	5.01 $\pm$ 1.25	4.71 $\pm$ 0.74
Serum HDL cholesterol (mmol/l)	1.02 $\pm$ 0.27	1.12 $\pm$ 0.23
Serum triglyceride (mmol/l)	2.27 $\pm$ 0.85	1.35 $\pm$ 0.37
Serum creatinine ($\mu\text{mol/l}$)	91.65 $\pm$ 11.41	87.00 $\pm$ 10.47
TNF- α score in subcutaneous adipocytes ^b	57	17
TNF- α score in visceral adipocytes ^b	40	11
Subcutaneous adipocyte cell volume (mm^3) ^c	0.00291 $\pm$ 0.00081	0.00253 $\pm$ 0.00064
Visceral adipocyte cell volume (mm^3) ^c	0.00278 $\pm$ 0.00076	0.00225 $\pm$ 0.00081***

* $P = 0.005$, ** $P < 0.0001$, *** $P = 0.037$ as compared with lean subjects.

^a OGTT 120 min value.

^b Visual evaluation (score) of the TNF- α protein in subcutaneous and visceral adipose tissue.

^c Adipocyte cell volume was calculated using the formula for ellipsoid body volume $[(a \times b^2) \times 4\pi/3]$. The shortest and longest diameters of 50 adipocytes were measured using a calibrated ocular (a and b are the long and short radius). The average was used for the mathematical calculation.

and compared. Adipocyte cell volume was determined using a graded ocular scale. Fat cell volume was calculated by determination of the longest and shortest cell diameter using the formula for an ellipsoid body $[(a \times b^2) \times 4\pi/3]$, where a and b are the long and short radius of the cell respectively. For the statistical analysis, the average of 50 cells from each sample was used.

Serum TNF- α (Sigma, St Louis, MO, USA; batch no. CKH-200A; mean intra-assay coefficient of variance (IACV): 4.8%, range: 4.2–5.3%; mean interassay coefficient of variance (IACV): 6.7%, range: 4.5–8.7%; lowest detectable concentration of recombinant human TNF- α : 0.25 pg/ml) and sTNFR-2 (BenderMedSystem, Vienna, Austria; cat. no.: BMS 211; IACV: 1.4%, range: 0.1–4.1%; IACV: 2.0%, range: 0.4–4.4%; lowest detectable concentration: 0.15 ng/ml) were determined by ELISA, and C-peptide was determined using RIA (Biodata, Rome, Italy; normal fasting range: 0.66–2.50 mg/ml; IACV: 5.6%, range: 4.3–6.1%; IACV: 7.3%, range: 5.8–8.1%; lowest detectable concentration: 0.2 ng/ml). HbA_{1c} measurements were performed by HPLC (normal range: 4.0–5.8%). Routine clinical laboratory parameters were measured using conventional laboratory techniques.

The Prism-3 program was used for the statistical analyses (Student's t -test, Mann–Whitney test, Fisher exact test, Spearman linear correlation coefficient) and graphical illustration of the results. The Shapiro–Wilk test was used for the analysis of the distribution of the data. A statistical program was used for the multiple regression analysis, to carry out partial correlations, and to adjust the subcutaneous and visceral cell volumes for BMI.

Results

Fat cell volume and expression of TNF- α in subcutaneous and visceral adipocytes of overweight/obese and lean persons

The subcutaneous (0.00291 ± 0.00081 vs 0.00253 ± 0.00064 mm³, mean $\pm$ s.d., NS) and visceral adipocyte cell volume (0.00278 ± 0.00076 vs 0.00225 ± 0.00081 , $P = 0.0362$, Mann–Whitney test) of overweight/obese subjects was greater than the lean ones (Fig. 1). The subcutaneous adipocytes were found to be slightly larger than the visceral ones, but no significant differences could be calculated in either subgroups (Table 1). TNF- α expression was detected in both fat depots in all patients in the overweight/obese subgroup (in 22 patients strong or moderate expression was observed). In the lean subgroup, TNF- α was detected in both fat depots in all except two patients, however the reaction was weaker (strong positivity was detected only in two subjects, $P = 0.0198$, compared with the overweight/obese subgroup, Fisher exact test). The total score detected in subcutaneous adipocytes was

Figure 1 Adipocyte cell volume of the subcutaneous and visceral depots in overweight/obese patients and lean controls. S.c. adipocyte cell volume (a), visceral adipocyte cell volume (b). n.s., non-significant; P value represents significant difference (Mann–Whitney test).

higher in both subgroups (overweight/obese: 57, lean: 17) as compared with visceral adipocytes (overweight/obese: 40, lean: 11, NS). Figure 2 represents adipocytes obtained from an overweight/obese patient with strong (subcutaneous, Fig. 2a) and weak (visceral, Fig. 2b) positivity.

Serum TNF- α , sTNFR-2 and C-peptide concentrations

Significantly elevated serum TNF- α (5.58 ± 0.87 pg/ml, mean $\pm$ s.d.) and sTNFR-2 (7.84 ± 3.56 ng/ml) concentrations were detected in the overweight/obese subgroup as compared with lean persons (4.21 ± 0.55 ; 4.59 ± 1.35 , $P < 0.01$, Mann–Whitney). Significantly higher serum TNF- α and sTNFR-2 levels were measured in patients with grades 2 and 3 TNF- α protein expression in their subcutaneous and visceral adipocytes (TNF- α : 6.06 ± 0.91 pg/ml, $P = 0.0008$; sTNFR-2: 7.49 ± 3.35 ng/ml, $P = 0.0098$, Mann–Whitney) as compared with those with grades 0 or 1 (TNF- α : 4.60 ± 0.83 , sTNFR-2: 5.29 ± 2.28).

Figure 2 Immunohistochemical staining of TNF- α protein in subcutaneous and visceral adipocytes ($\times 200$). Adipocytes with strong staining (3+ positivity) from subcutaneous fat cells of an obese patient (a) and weak positivity (1+ positivity) in visceral adipocytes of a lean person (b) are shown.

Similarly, both fasting (2.91 ± 1.20 ng/ml) and post-challenge C-peptide concentrations (10.02 ± 3.73) were significantly ($P < 0.01$) higher in the overweight/obese subgroup as compared with the lean persons (1.43 ± 0.58 ; 1.98 ± 0.68). Another indirect parameter of insulin resistance, the fasting C-peptide/blood glucose ratio, was also significantly greater (0.53 ± 0.21) in the overweight/obese subgroup as compared with the lean probands (0.28 ± 0.10 , $P < 0.01$, Mann-Whitney). The post-challenge (OGTT, 120 min) C-peptide/blood glucose ratio was also greater in the overweight/obese subgroup (1.39 ± 0.52 vs 0.29 ± 0.10 , $P < 0.01$) (Table 1).

Correlations between subcutaneous and visceral adipocyte cell volume, and serum TNF- α , sTNFR-2 and C-peptide concentrations

Significant positive linear correlations were found between subcutaneous and visceral adipocyte cell volume, and serum TNF- α , sTNFR-2 and C-peptide

Figure 3 Correlation between subcutaneous adipocyte cell volume and (a) serum TNF- α ($n = 43$), (b) serum sTNFR-2 and (c) fasting C-peptide concentrations in the subjects studied. (Linear correlation, Spearman; r = correlation coefficient, P = value of significance).

levels in the total group of patients (Fig. 3), and in the overweight/obese subgroup (Fig. 4, Table 2). In the overweight/obese group the grade of the TNF- α expression in the subcutaneous ($r = 0.58$, $P = 0.006$) and visceral adipocytes ($r = 0.46$, $P = 0.042$) correlated with the serum TNF- α concentrations. Moreover, BMI and the C-peptide/blood glucose ratio also correlated with the cell volume from both fat depots. In the lean subgroup only serum TNF- α and sTNFR-2 correlated with the subcutaneous and visceral cell volumes. No correlations were found between C-peptide levels, C-peptide/blood glucose ratio and cell volumes

Figure 4 Correlation between visceral adipocyte cell volume and (a) serum TNF- α , (b) serum sTNFR-2 and (c) fasting C-peptide concentrations in the subjects studied. (Linear correlation, Spearman; r = correlation coefficient, P = value of significance).

in the lean persons (Table 2). The post-challenge C-peptide/blood glucose ratio correlated positively with the visceral fat cell volume ($r = 0.47$, $P = 0.0001$, Spearman) in all patients, and in the overweight/obese subgroup ($r = 0.52$, $P = 0.004$). No correlation was found between the post-challenge C-peptide/blood glucose ratio and the subcutaneous adipocyte cell volume in either subgroup.

Multiple linear regression analysis of the studied parameters

Adjusting the subcutaneous and visceral cell volume for BMI, multiple linear regression analysis was performed

Table 2 Correlations between laboratory parameters and adipocyte cell volume in overweight/obese patients and lean probands.

	BMI	TNF- α	sTNFR-2	C-pf	C-pf/BGf	scCV	viscCV
BMI							
Obese r	0.51	0.53	0.42	0.35	0.57	0.40	
P	0.003	0.001	0.012	0.031	0.001	0.018	
Lean r	0.11	0.04	0.12	0.21	0.64	0.11	
P	NS	NS	NS	NS	0.004	NS	
TNF-α							
Obese r		0.25	0.49	0.47	0.58	0.75	
P		NS	0.003	0.005	0.001	0.001	
Lean r		0.10	0.25	0.15	0.46	0.52	
P		NS	NS	NS	0.041	0.022	
sTNFR-2							
Obese r			0.74	0.70	0.49	0.33	
P			0.001	0.001	0.003	0.043	
Lean r			0.16	0.20	0.47	0.55	
P			NS	NS	0.043	0.016	
C-pf							
Obese r				0.78	0.44	0.35	
P				0.001	0.008	0.035	
Lean r				0.75	0.25	0.26	
P				0.001	NS	NS	
C-pf/BGf							
Obese r					0.32	0.27	
P					0.050	NS	
Lean r					0.13	0.18	
P					NS	NS	
scCV							
Obese r						0.60	
P						0.001	
Lean r						0.39	
P						NS	

Each correlation is represented only once. C-pf, fasting C-peptide level; C-pf/BGf, fasting C-peptide/blood glucose ratio; scCV, subcutaneous adipocyte cell volume; viscCV, visceral adipocyte cell volume. r = Spearman's coefficient of correlation. P = value of significance, NS, non-significant correlation.

to evaluate their correlations with serum TNF- α , sTNFR-2 and C-peptide concentrations in the total group. By this approach concerning the subcutaneous adipocyte cell volume, the correlation with sTNFR-2 concentration remained significant ($\beta = 0.556$, $P = 0.005$). No correlations with TNF- α ($\beta = 0.301$, $P = 0.173$) or C-peptide levels ($\beta = 0.198$, $P = 0.285$) were detected. In contrast, the visceral adipocyte cell volume corrected for BMI remained in a significant positive correlation with TNF- α ($\beta = 0.797$, $P = 0.0007$) and sTNFR-2 ($\beta = 0.457$, $P = 0.005$). The correlation between the visceral fat cell volume and fasting C-peptide concentration was close to significance ($\beta = 0.327$, $P = 0.074$).

Discussion

The adipose tissue expression of TNF- α mRNA has already been described in rodent models of obesity and also in humans (2, 13). Subcutaneous fat depots exhibited 1.67-fold higher TNF- α mRNA expression

as compared with the omental ones (2). The amount of TNF- α mRNA was found to correlate positively with adiposity, and decreased after weight loss (6). Moreover, TNF- α mRNA expression was also reported to correlate with fasting hyperinsulinemia (14).

In this study, the expression of TNF- α protein was investigated in overweight/obese and lean subjects. In accordance with the above-mentioned observations, we detected a slightly higher amount of TNF- α protein in the subcutaneous adipose tissue as compared with the visceral fat cells in both subgroups. By using a visual semi-quantitative evaluation of the immunostaining, an increased TNF- α protein expression could be observed in both fat depots of the overweight/obese patients. The calculated subcutaneous and visceral cell size correlated with the BMI value, serum TNF- α and sTNFR-2 concentrations in the total patient group and in the overweight/obese subgroup.

The fasting and post-challenge C-peptide concentrations, as well as the C-peptide/blood glucose ratios can be considered as easily measurable indirect parameters representing the insulin resistance (4, 15). In our study, serum TNF- α and sTNFR-2 concentrations and the subcutaneous and visceral adipocyte cell volume were found to be in a significant positive linear correlation with these parameters in all patients as well as in the overweight/obese subgroup.

In the light of these observations, we suggest that the cell volume in both adipocyte depots can be a determinant of the expression and production of both TNF- α and sTNFR-2. These data are in accordance with the results of Skoog *et al.*, who reported elevated fat cell volume and increased *ex vivo* TNF- α production in obese patients (16). Our observations on the connections between TNF- α , sTNFR-2 and indirect parameters of insulin resistance in the overweight/obese patients support the role of the overexpression of the TNF system in obesity-linked insulin resistance.

It is generally accepted that the unfavorable metabolic effects of adiposity are mostly due to the increased visceral fat depot. Somewhat surprisingly, in contrast to this prevailing concept, we have observed a slightly higher expression of the TNF- α protein in the subcutaneous depot, in the total group, as well as in both subgroups. Moreover, the subcutaneous fat cell volume was found to correlate with measured and calculated indirect parameters of insulin resistance at least to the same extent as the visceral fat cell volume. Therefore, these results may underline the concept about the functional plasticity of adipocyte depots. Moreover, several other adipocyte-derived cytokines and transcription factors – such as leptin, resistin, adiponectin, interleukin-6 and PPAR- γ – may also influence TNF- α production of the fat cells and insulin sensitivity; some of them also exhibit depot-specific differences (17–19). It can be hypothesized that the mutual interaction of these components may explain the functional plasticity of the subcutaneous fat cell

depot. This concept is supported by the observation that after the administration of thiazolidine-dione drugs – external ligands of peroxisome proliferator activated receptor (PPAR)- γ nuclear receptors – a more pronounced increase of the subcutaneous fat depot was found as compared with the visceral fat depot (20). In addition, in humans the expression of PPAR- γ , one of the known suppressors of TNF- α gene expression, was found to be increased in obese subjects in their visceral fat compartment as compared with their subcutaneous adipose depot (21).

In conclusion, the novelty of this observation is the expression of the TNF- α protein in both fat depots and the correlation of serum TNF- α and sTNFR-2 with the adipocyte cell volume. Thus, the adipocyte cell volume of both the subcutaneous and visceral fat depots may be determinants of TNF- α and sTNFR-2 production and insulin resistance.

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