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Level of *Ex Vivo* Interleukin 6 Expression in Human Peripheral Fat Compared with Other Tissues

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Objectives. *Adipose tissue is able to secrete a variety of active mediators into the circulation. One of these is Interleukin 6 (IL6). IL6 may play a causal role in the development of atherosclerosis. It has therefore been suggested that IL6 may form part of the link between obesity and vascular disease. The aim of this study was to quantify the relative IL6 expression in adipose tissue compared to other tissues.*
Methods. *Tissue (vein, fat, muscle, blood) was collected from 32 patients undergoing varicose vein surgery. RNA was extracted and mRNA measured using RT-PCR relative quantification. The mean relative IL6 mRNA levels were compared between tissues using the Mann Whitney U test and the independent t-test. Tissue levels were compared for individuals using the Wilcoxon signed rank test.*
Results. *Mean relative IL6 mRNA levels (mean ± SEM) were significantly greater in adipose tissue 44.8 ± 16.1 than in other tissues (leukocytes 1.1 ± 0.3, vein 2.0 ± 0.8, muscle 0.06 ± 0.03:* $p < 0.001$*). mRNA expression levels were also significantly higher in fat than in all other tissue types in individuals (*$p < 0.001$*).*
Conclusions. *IL6 mRNA expression is significantly higher in adipose than in many other tissues known to express IL6.*

Keywords: Interleukin 6; Atherosclerosis; Obesity; Adipose tissue; mRNA expression.

Introduction

Atherosclerosis is the most common cause of death in the western world.[1] Obesity, a major risk factor for arterial disease, has undergone a rapid increase in prevalence in recent years.[2,3] This may result in an increased incidence of atherosclerosis in the future.[4] It is therefore important to explore the mechanisms underlying the association of obesity and vascular disease.

Interleukin 6 (IL6) is a pro-inflammatory cytokine. Raised circulating IL6 levels are associated with arterial disease and as well as arterial disease progression.[5,6] Levels of plasma IL6 correlate with sub-clinical atherosclerosis[7] and appear to predict future risk of cardiovascular events.[5] These findings have led to the hypothesis that IL6 may form part of the causal chain in arterial disease development and progression. In addition, IL6 may influence outcome from surgical treatment as raised levels of IL6 are associated with an increased systemic inflammatory response and risk of multi-organ failure following aortic surgery.[8] IL6 may further play a role in the long term outcome from vascular surgery determined by the development of intimal hyperplasia leading to graft failure.[9]

It has become apparent that adipose tissue has an important endocrine function and is able to release a variety of active mediators into the circulation,[10] one of them IL6.[11] Plasma levels of IL6 are positively associated with body mass index[12–14] and adiposity and decrease with weight loss.[12,14,15] It has been suggested that obesity represents a low grade inflammatory state and that the associated increase in circulating IL6 may affect the vascular endothelium, explaining the link between obesity and atherosclerosis.[16–19]

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IL6 is expressed in a variety of tissues apart from adipose tissue including muscle, vascular wall and blood.[20] It is unclear to what extent the individual tissues contribute to the total circulating IL6 levels. We thus sought to quantify IL6 mRNA in tissues known to produce IL6 (adipose tissue, vein, skeletal muscle and leukocytes), in a series of human subjects. Subjects suffering from vascular disease were excluded, as atheromatous disease itself can affect IL6 RNA levels in certain tissues[21] and could therefore mask or exaggerate the effect of tissue type on gene expression.

Methods

Subjects

Following ethic consent (Southampton & South West Hampshire Research Ethics Committees (REC) Reference: 040/04/w), 32 consecutive subjects with American Society of Anaesthesiologists (ASA) grades I or II who were due to undergo routine varicose vein surgery were recruited to the study. Excluded were those with a history of arterial, malignant or acute inflammatory disease and those currently taking steroids. Patient age, body mass index, ethnic origin, past medical history, medication and smoking habits were documented.

Tissue collection and RNA extraction

Vein, blood, skeletal muscle (pectineus) and peripheral adipose tissue (128 tissue samples) were collected from all subjects. Tissues were immediately submerged in RNA*later*® (Sigma-Aldrich) to maintain the *in vivo* RNA profile. Blood samples were collected using PAXgene™ Kit (PreAnalytiX).

RNA was extracted from all the collected tissues using a modified TRI-Reagent® (Sigma-Aldrich) method. Leukocyte RNA was extracted using the PAXgene™ Kit. RNA was quantified using spectrophotometry. For reverse transcription, 500 ng of total RNA was transcribed into cDNA using random hexamer primers (PrimerDesign Ltd, http://www.primerdesign.co.uk/) and M-MLV reverse transcriptase (PrimerDesign Ltd, http://www.primerdesign.co.uk/).

Real time PCR analysis

Real time polymerase chain reaction (PCR) analysis was used to measure IL6 mRNA expression in 128 samples using the LightCycler® 480 Real Time PCR System. The IL6 assay consisted of pre-designed primers and fluorescein labelled (FAM) TaqMan probe and quencher (AppliedBiosystems: Hs00174131_m1). Analysis was performed as relative quantification with reference to the housekeeping gene glyceraldehye-3-phosphate dehydrogenase (GAPDH). The assay for GAPDH used pre-designed primers and a FAM hydrolysis probe and quencher (Universal Probe Library Probe #60, Roche). Both target and housekeeping assays were cDNA specific. Relative quantification data was analysed using ROCHE LC480® Relative Quantification Software. This software uses a standard curve to account for differences in efficiency between reference and target gene assay and allows normalisation to a calibrator sample to account for differences between individual runs. Reactions were performed in duplicate with a standard curve in each run.

Statistical analysis

Only samples with relative quantification data for all four tissue types were included in the statistical analysis. Statistical analysis was performed using 'statistical package for the social sciences' (SPSS) software. A p-value of less than 0.0125 was considered significant. The normalised concentration ratios were grouped together according to tissue type. The normalised concentration ratio was not normally distributed in any of the tissues. Therefore to test for significant difference in expression between tissue types, the Mann-Whitney U test was applied. The data was also logarithmically transformed allowing reanalysis using the independent t-test with equal variance not assumed.

Normalised concentration values in the four tissues were then matched for each subject and variation of IL6 expression between tissues was assessed for the individual patient by Wilcoxon signed rank test.

To test for a correlation between IL6 expression in adipose tissue and BMI the Spearman rank correlation was calculated.

Results

Patients all were of European origin. Out of the 32 recruited patients, normalised concentration ratios were available for all four tissues in 21 patients i.e. 84 tissue samples. Out of the 21 subjects (12 female, 9 male) five were cigarette smokers and ten took regular medication: nifedipine, hydrochlorothiazide, aspirin, salbutamol, omeprazole, allopurinol were taken by one patient each. Two patients took amitriptyline and

two took an oral contraceptive pill. The average age of our patients was 47 years (range 24–69 years).

Expression of IL6 mRNA in adipose tissue was significantly higher in adipose tissue than in all other tissue types examined (Table 1 & Fig. 1). The mean ± SEM normalised concentration ratio of IL6 compared with the housekeeping gene GAPDH was 44.8 ± 16.1 (median = 14.9) for adipose tissue; 1.1 ± 0.3 (median = 0.8) for leukocytes; 2.0 ± 0.8 (median = 0.4) for vein and 0.06 ± 0.03 (median = 0.03) for muscle. Median and mean levels of IL6 mRNA were significantly higher in adipose tissue than in all other tissues (Mann Whitney U: (two-tailed) $p < 0.001$; independent t-Test: (two-tailed) $p \leq 0.001$).

In the individual subject, levels of IL6 mRNA were also significantly higher in fat when compared to all other tissues for the same subject (Wilcoxon signed rank test $p < 0.001$) (Fig. 2). IL6 mRNA level in adipose tissue was on average 55.0, 35.8 and 697.3 times higher than IL6 expression in leukocytes, vein and muscle respectively in the same individual.

BMI was recorded in 20 patients. The mean ± SEM body mass index (BMI) was 27.3 ± 0.9. There was no significant correlation between IL6 expression in adipose tissue and BMI (Spearman correlation coefficient = 0.24; significance (2-tailed) = 0.31) i.e. increase in BMI did not associate with an increase in relative IL6 RNA expression or vice versa (Fig. 3).

Table 1. Relative levels of interleukin 6 mRNA in four different tissues in 21 patients

Patient identification number	Normalised concentration ratio by tissue type			
	Adipose	Leukocytes	Vein	Muscle
10	14.90	1.49	0.40	0.01
13	6.50	0.97	0.21	0.05
14	2.49	0.76	0.24	0.00
15	108.00	1.81	7.56	0.03
17	98.16	0.72	0.82	0.02
18	303.00	6.11	15.52	0.72
19	13.23	0.40	5.21	0.09
24	38.42	0.47	0.38	0.04
25	6.17	1.37	1.63	0.04
26	11.97	1.39	0.29	0.02
28	174.00	0.46	0.66	0.02
29	27.61	0.83	0.24	0.04
30	21.85	1.52	4.34	0.08
31	2.38	0.60	1.28	0.02
34	11.16	1.53	0.22	0.00
35	5.80	0.90	1.65	0.00
36	16.63	0.66	0.64	0.04
37	4.60	0.64	0.16	0.01
38	57.38	0.24	0.00	0.04
41	15.07	0.35	0.23	0.03
43	2.48	0.85	0.41	0.05

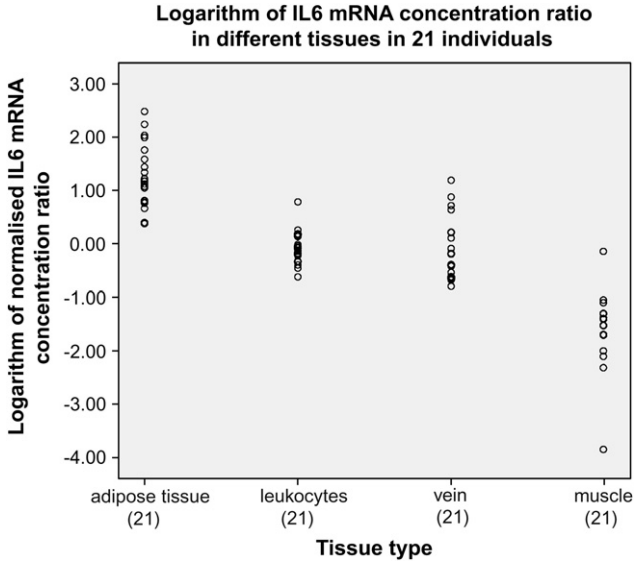

Fig. 1. Individual values for the logarithm of the normalised concentration ratio of IL6 expression in different tissues. There are 21 samples represented in each tissue type.

Discussion

To date, comparative data on IL6 expression has been available in the form of the gene expression atlas.[22] However vein, muscle and adipose tissue are not represented in the atlas. Furthermore, the gene

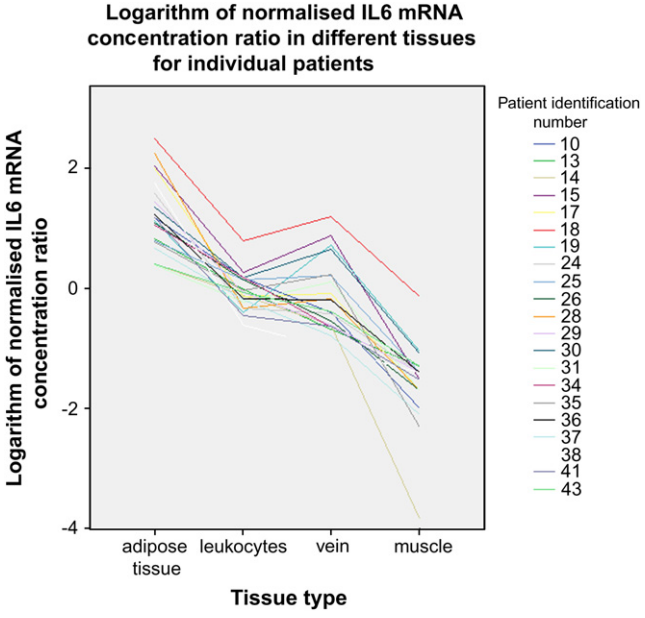

Fig. 2. Logarithm of IL6 mRNA normalised concentration ratio for individual patients in all four tissue types. In every patient adipose tissue shows the highest values for IL6 mRNA levels.

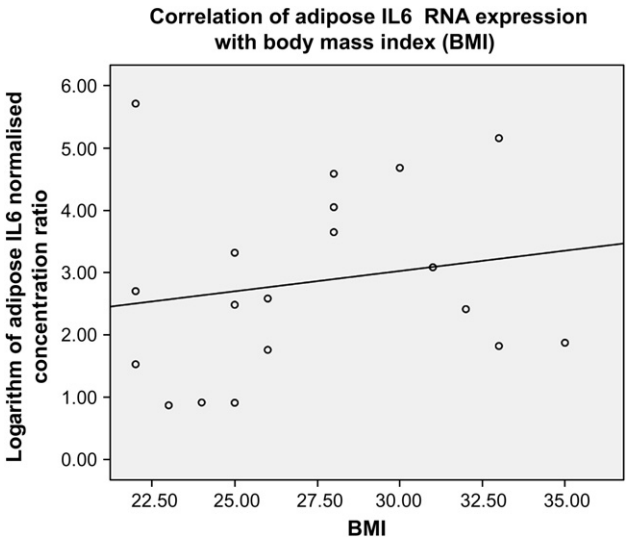

Fig. 3. Graph showing correlation of adipose IL6 RNA expression with body mass index. The trend line shows a very weakly positive correlation which is not significant ($p = 0.32$).

expression atlas data is based on commercially obtained tissues which originate from different individuals. There is large variation in IL6 expression in individuals, partially related to variables such as smoking, medication and age, which can affect the result.[23–25] This study uses paired tissue samples from one individual, taken at the same time point thereby reducing the effect of these confounding variables giving a more accurate insight into the effect of tissue type on IL6 gene expression.

Our data shows that adipose tissue expresses significantly more IL6 mRNA than the other tissues examined, suggesting that increased adiposity might influence circulating IL6 levels and thus vascular risk and treatment outcome. These data are consistent with a 23% fall in plasma IL6 levels associated with 33% weight loss in the very obese.[15] Indeed, arteriovenous differences in plasma IL6 levels across an abdominal subcutaneous tissue bed suggest that up to 30% of plasma IL6 may be derived form adipose tissue depending on BMI.[26] This study measured IL6 RNA expression in adipose tissue relative to a housekeeping gene rather than plasma IL6 levels. Therefore the lack of correlation between BMI and adipose tissue expression is not surprising, as the increase in plasma IL6 seen in obesity is likely to relate to increase in total body fat mass. However, it is possible that obesity further leads to increased IL6 expression through a change in the cell composition of adipose tissue with a relative increase in those cells that are mainly responsible for the expression of IL6. Only 10% of IL6 derived from adipose tissue cell culture arises from adipocytes.[27] Other sources of IL6 in adipose tissue are stromal-vascular cells and macrophages, with macrophages thought to be the main contributors.[28,29] Adipose tissue macrophage infiltration is increased in obese subjects[30] and decreases during weight loss.[31] Therefore the relative IL6 expression in adipose tissue may increase with adiposity beyond a pure volume effect. This study does not support this view, as there was no significant correlation between BMI and IL6 expression in adipose tissue. However, correlation was only tested in 20 samples. It is possible that variables such as age, smoking or medication masked any effect of cell composition on adipose IL6 expression. Further studies may provide better insight.

A number of patients had considerably higher IL6 expression compared to other subjects in some tissues. Possible reasons include unidentified genetic variables or variables such age, smoking, medication or undiagnosed infection at the time of sample collection. The design of the study aimed to reduce the influence of such variables on the outcome measure of relative tissue expression by performing *intra*-individual comparison i.e. all four samples came from the same individual, therefore all samples were subject to the same unidentified variable. However, it can not be excluded that some variables may affect individual tissues differently. Investigating potential causes for higher expression in some patients requires an *inter*-individual comparison which may be difficult to interpret in a relatively small sample set. Such a comparison was therefore not performed.

It is not possible to deduce the exact percentage adipose tissue contributes to the circulating IL6, from relative IL6 mRNA levels measured in this study. Various steps exist that exert post transcriptional control, i.e. not all mRNA will be translated into protein, and these post-transcriptional changes may vary between tissue types. In addition, protein production within the cell may not translate into equivalent secretion of the protein, as an intracellular signalling pathway for IL6 is thought to exist.[32] It is therefore possible that a proportion of IL6 will never leave the cell. In addition, some of the IL6 secreted by adipose tissue may act in a paracrine manner and therefore remain within the tissue rather than enter the circulation, though release of IL6 *has* been demonstrated from peripheral fat.[17] Despite these reservations, it is thought that the circulating levels of IL6 are mainly regulated at the level of gene expression,[33] making it likely that the difference in mRNA expression will be reflected in tissue contribution to IL6 plasma levels.

Adipose tissue expressed forty times as much IL6 mRNA as leukocytes in this study. However the percentage contribution of adipose tissue to plasma IL6 levels depends on levels of adiposity, thus its' relative importance is decreased in lean subjects. In addition IL6 secreted by circulating leukocytes will immediately contribute to IL6 plasma levels unlike adipose derived IL6 which may remain within the tissue itself. Thus leukocyte derived IL6 may play a more important role in plasma levels then the figures for mRNA expression suggest.

In this study the mean mRNA expression in fat tissue was almost 700 times greater than the expression in muscle. The transcription of IL6 in muscle as well as net release from muscle has been shown to increase following exercise.[34,35] Although exercise also appears to increase IL6 expression in adipose tissue, the increase is relatively smaller than the one observed in muscle. One would expect exercise to lead to a relatively higher increase in contribution from muscle to circulating IL6 levels.[36] In addition, it has been shown that IL6 release from muscle increases from the fasting level following a high fat meal. In this study patients presented to hospital following overnight rest and overnight fast. The relative level of IL6 expression in muscle in more active subjects and subjects in the postprandial phase may therefore be higher than demonstrated here.

It is known that visceral obesity constitutes a greater risk factor for atherosclerosis than peripheral adiposity.[37] Studies examining site of production of circulating IL6 suggest a greater contribution from visceral fat than from peripheral adipose tissue.[27,38] This study examined subcutaneous fat samples only. It is likely that the relative contribution of visceral fat to systemic IL6 is higher than the figures presented here, emphasising the importance of weight loss in decreasing circulating levels of IL6.

Raised circulating IL6 levels are associated with the development and progression of vascular disease as well as negative outcome from surgery both in terms of short term outcome and long term vascular graft patency. The findings from this study suggest that increase in adipose tissue mass has a distinct potential to increase circulating IL6 levels, as adipose IL6 expression appears to be significantly higher than expression in other tissues. Assessing whether this forms a causal link between obesity and vascular disease, requires further research into the IL6/vascular disease relationship. Studies utilising genetic variations in the *IL6* gene are effective tools for exploring the causal nature of the association between mediator and disease. Such studies are likely to advance our knowledge in the future.

Conclusion

IL6 mRNA levels in human subjects are significantly greater in adipose tissue than in other tissues known to express IL6. It is therefore likely that IL6 expression from adipose tissue has important paracrine, endocrine or inflammatory roles.

Conflict of interest

Work in the laboratory of Ian Day is funded by UK MRC and British Heart Foundation.

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