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Tumour necrosis factor alpha as a predictor of impaired peak leg blood flow in patients with chronic heart failure

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Summary

Tumour necrosis factor alpha (TNFα) is increased in patients with cardiac cachexia, a condition associated with reduced peripheral blood flow both at rest and after interventions causing vasodilation. By contrast, in patients with chronic heart failure (CHF), higher TNF levels are associated with a greater capacity for vasodilation in the arm. To clarify the relationship between peripheral blood flow and TNF in CHF, we studied the relation between TNFα and blood flow in the leg (plethysmography, post maximal exercise and 5 min ischaemia) in 34 patients (age 63 $\pm$ 2 years, ejection fraction 29 $\pm$ 3%, peak VO_2 16.6 $\pm$ 1.1 ml/kg/min, mean $\pm$ SEM). Peak leg blood flow correlated significantly with total TNFα ($r = -0.68$, $p < 0.0001$), peak VO_2 ($r = 0.54$), and soluble TNF receptors 1 ($r = -0.56$) and 2 ($r = -0.52$, all $p < 0.002$). TNFα, soluble TNF receptors 1 and 2 and aldosterone correlated with peak blood flow independently of age, ejection fraction, peak VO_2 and functional NYHA class. TNFα was the only parameter that showed strong correlations for peak blood flow in all clinically relevant subgroups (severe vs. mild, ischaemic vs. dilated, cachectic vs. non-cachectic patients). This study shows a close and inverse relationship between peak leg blood flow and the plasma concentration of TNFα, suggesting a pathophysiological role for TNFα in reducing peak peripheral blood flow in CHF.

Introduction

Reduced peripheral blood flow in patients with chronic heart failure (CHF) is attributed to a reduced cardiac output, and to excessive vasoconstrictor tone. The reduced peripheral blood flow is linked to the symptoms and clinical features of patients with heart failure.[1] Significant correlations between peak limb blood flow and exercise capacity (maximal oxygen consumption (peak VO_2), have been demonstrated in these patients,[1,2] but not consistently.[3,4] We have shown previously that peak leg blood flow predicted peak VO_2 only in CHF patients with cachexia.[5]

In 1990, Levine *et al.*[6] demonstrated elevated plasma levels of tumour necrosis factor (TNFα) in patients with severe CHF, especially in cachectic patients. This was confirmed by other groups.[7,8] TNFα is a key cytokine for the initiation of catabolism.[9] It was recently reported[10] that patients with CHF and high TNFα levels had higher post-ischaemic forearm blood flows and higher blood flow responses to administration of acetylcholine and nitroglycerin; these observations were attributed to increased activation of inducible NO synthase.[10] The findings have not yet been confirmed in other clinical studies, but there is *in vitro* evidence for the suggested mechanism of increased NO production (reviewed in reference 11). This is surprising, as healthy subjects

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have low TNFα levels but high peak blood flows, whereas patients with severe CHF have elevated TNFα levels but reduced peripheral blood flow. The objective of the present study was, therefore, to clarify the relation between peak blood flow and cytokines in patients with CHF, taking into account disease severity and factors that are known to influence peripheral vasodilation. The studies on blood flow were made in the leg rather than the arm.

Methods

Patients

Thirty-four men (age 62.6 ± 2.0 years, range 34–83 years, mean ± SEM) with stable heart failure of at least 6 months duration were investigated (left ventricular ejection fraction by nuclear scan: 29 ± 3%). All studies were performed within a maximum of 2 days, arranged so that the blood samples were always taken in the fasting state between 9 and 10 am. Two patients were in functional New York Heart Association (NYHA) class I, 13 in class II, 15 in class III, and 4 in class IV. Sixteen patients had signs of clinical cachexia, whereas 18 patients were non-cachectic. Patients with clinical cachexia were defined as those with a documented weight loss of $\geqslant$7.5% of previous normal weight (range 8–30%) over a period of $\geqslant$6 months.[12] The patients were on stable medication for at least 4 weeks prior to the study. The patients were being treated with diuretics (n=31), ACE inhibitors (26), aspirin (12), warfarin (11), digitalis (10), oral nitrates (9), and calcium antagonists (2) in varying combinations. No patients had signs of renal failure, acute infection, peripheral or pulmonary oedema, hepatomegaly or ascites. All patients gave written informed consent. The protocol was approved by the Ethics Committee of the Royal Brompton Hospital, London.

Blood samples

Standard venous blood samples were obtained after overnight fasting and 20 min supine rest. After centrifugation, aliquots were stored at −70 °C until analysis. Human growth hormone (hGH), insulin-like growth factor-1 (IGF-I), and aldosterone were measured using radio-immunoassays.[13] TNFα was measured using an immunoassay with a lower limit of detectability of 3.0 pg/ml (Medgenix). The latter is an ELISA test using three antibodies directed against distinct epitopes of TNFα that is not influenced by soluble TNF receptors.[14]

Additionally, we investigated the levels of soluble TNF receptor-1 (sTNFR1), sTNFR2, interleukin-1β (IL-1β) and IL-6 by ELISA.[15] In a subset of 26 patients, we were able to perform an additional TNFα assay using the Quantikine high sensitivity (0.5 pg/ml) kit from R&D Systems to validate our results. This test kit measures intact TNF trimers, and is also not influenced by soluble TNF receptors. The cytokines as well as hGH, IGF-I, insulin and aldosterone, were measured in duplicate. Unless otherwise stated, data in this paper refer to total TNF levels measured with the Medgenix kit. All other parameters were analysed by the routine analysis of our hospital.

Functional tests

Maximal oxygen consumption (peak VO_2 in ml/kg/min) was measured by treadmill exercise testing.[3] Ultrafast computerized tomography (Imatron) was used to determine the muscle cross-sectional area of the right thigh transaxially at mid-femur level.[3] Leg blood flow in the right leg was determined using venous occlusion plethysmography (EC4, Hokanson) after at least 10 min rest, and peak flow after exercise and an additional 5 min ischaemia.[4]

Statistical analysis

Results are presented as means ± SEM. We used simple linear regression to analyse the predictors of peak leg blood flow (StatView 4.5, Abacus Concepts). As 30 clinical and biochemical/haematological parameters were analysed, a probability value of $p<0.00167$ was considered statistically significant (Bonferroni correction). Subgroup analyses were subsequently only performed for those factors that fulfilled these criteria *and* correlated independently of age. Multivariate analysis and stepwise regressions were also performed and here $p<0.05$ was considered statistically significant.

Results

Physiological tests

The CHF patients had a mean peak VO_2 of 16.6 ± 1.1 ml/kg/min. The cross-sectional area of the right thigh muscles amounted to 103.3 ± 4.0 cm^2. The right leg blood flow at rest was 2.9 ± 0.2 ml/100ml/min (range 1.05–5.76 ml/100ml/min), increasing to a peak of 24.1 ± 1.3 ml/100 ml/min (range 7.89–37.50 ml/100ml/min). The results of the blood tests are presented in Table 1.

Peak leg blood flow correlation

There was a significant univariate correlation between peak leg blood flow and peak VO_2 (r = 0.54, p=0.001). Due to the chosen significance level, age (r=0.47, p=0.005), BMI (r=0.37,

Table 1 Biochemical and haematological characteristics of 34 chronic heart failure patients

	Mean ± SEM	Range
Sodium (mmol/l)	137 ± 3	130–142
Potassium (mmol/l)	3.90 ± 0.08	3.00–5.20
Plasma cholesterol (mmol/l)	5.58 ± 0.23	2.59–9.05
Plasma HDL cholesterol (mmol/l)	1.15 ± 0.07	0.55–2.53
Plasma LDL cholesterol (mmol/l)	3.69 ± 0.20	1.35–6.42
Plasma glucose (mmol/l)	5.88 ± 0.37	4.56–16.88
Plasma insulin (pmol/l)	56.7 ± 7.1	6.0–223.6
Haemoglobin (g/dl)	13.0 ± 0.3	10.3–15.3
WCC ($\times 10^9$/l)	7.1 ± 0.4	2.9–10.6
Plasma albumin (g/l)	43.6 ± 0.5	36–50
Plasma aldosterone (pmol/l)	533 ± 65	94–1519
Human growth hormone (ng/ml)	3.03 ± 0.86	0.02–23.20*
Insulin-like growth factor-1 (nmol/l)	130.6 ± 8.7	72.8–266.6
Plasma renin activity (ng/ml/h)	13.3 ± 2.7	0.67–63.82
Thyroid-stimulating hormone (mU/l)	2.27 ± 0.29	0.62–8.07
Plasma adrenaline (nmol/l)	2.07 ± 0.32	0.12–5.40
Plasma noradrenaline (nmol/l)	4.27 ± 0.48	1.18–11.94
Total TNF-α (pg/ml) (Medgenix)	12.21 ± 1.82	3.00–56.16**
sTNF receptor 1 (pg/ml)	1476 ± 162	485–4788
sTNF receptor 2 (pg/ml)	2552 ± 160	1263–5232
Interleukin-1β (pg/ml)	0.278 ± 0.055	0.010–0.949***
Interleukin-6 (pg/ml)	3.58 ± 0.54	0.44–11.03

* Three patients had levels below 0.02 ng/ml (limit of detectability). ** Four patients had levels below 3.00 pg/ml (limit of detectability). *** Seven patients had levels below 0.01 pg/ml (limit of detectability). (For these parameters a value at the limit of detectability was recorded for statistical analyses.)

$p<0.05$), total area of the thigh muscles ($r=0.41$, $p<0.01$) and right thigh cross-sectional area ($r=0.36$, $p<0.05$) did not correlate significantly with peak leg blood flow. Three cytokines correlated significantly and inversely with peak leg blood flow: TNFα ($r=-0.68$, $p<0.0001$, Figure 1), sTNFR1 ($r=-0.56$, $p=0.0005$), and sTNFR2 ($r=-0.52$, $p=0.0016$). There were trends for a correlation between peak blood flow and hGH ($r=-0.48$, $p=0.004$) and aldosterone ($r=-0.41$, $p<0.05$), but no significant correlations with left ventricular ejection fraction ($r=0.08$), NYHA class ($r=-0.19$), or any of the other measured biochemical or haematological factors.

On multivariate analysis, TNFα ($p<0.0001$), sTNFR1, aldosterone (both $p=0.006$), sTNFR2 ($p=0.02$), hGH, and peak VO_2 (in ml/kg/min, both $p=0.04$) correlated with peak leg blood flow independently of age. The following factors also correlated independently of ejection fraction, NYHA class and peak VO_2: TNFα ($p=0.001$), sTNFR1 ($p=0.006$), aldosterone ($p=0.02$) and sTNFR2 ($p=0.03$). In multivariate analysis with only the latter four factors, TNFα was the only independent factor ($p=0.04$). A stepwise model with clinical and haematological/biochemical factors also found TNFα to be the strongest predictor of peak blood flow ($r=-0.68$, $r^2=0.463$, $p<0.0001$, F-value 27.56). After

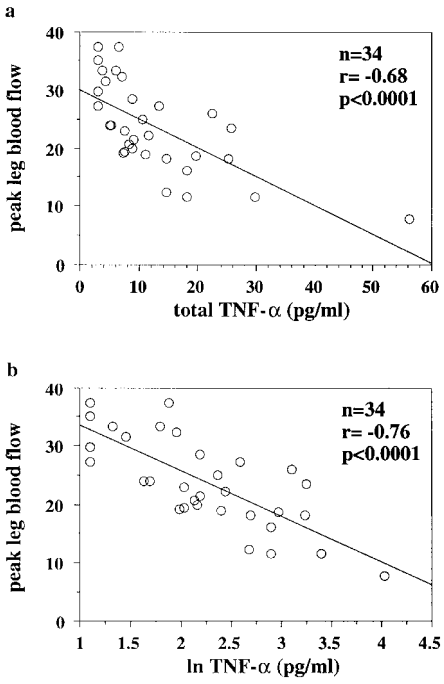

Figure 1. The relation between peak leg blood flow and TNFα in 34 patients with chronic heart failure. **a** Total TNFα vs. peak leg blood flow ($y=30.168-0.497x$).—analysis without one outlying result: $r=-0.63$, $p<0.0001$. **b** Logarithmic transformation of total TNFα levels vs. peak leg blood flow ($y=41.5-7.836x$).

adjusting for TNFα, only hGH (F-value 6.98) and age (F-value 4.46) were significantly related to peak blood flow. The best two-step model was: TNFα and hGH together vs. peak leg blood flow: $r = -0.75$, $r^2 = 0.561$, $p < 0.0001$. The principal results were confirmed using a second test kit for the assessment of TNFα (R&D systems, data not shown). To analyse whether the observed correlations were related to a specific group of heart failure patients, the study population was subgrouped by NYHA class, the presence of cachexia, and the disease aetiology. Only total TNFα ($r = -0.50$ to -0.74) was a strong predictor of peak leg blood flow in all analysed subgroups (data not shown).

Correlates of TNFα

Simple regression analysis showed significant inverse correlations between TNFα and peak leg blood flow (Figure 1), leg muscle size ($r = -0.55$, $p = 0.0007$) as well as peak VO_2 ($r = -0.54$, $p = 0.0009$), and a positive relation to sTNFR1 and sTNFR2 (both $r = 0.67$, $p < 0.0001$).

Discussion

The principal finding of this study is that TNFα is significantly and inversely related to peak leg blood flow in patients with CHF. This relation is seen in patients with CHF independently of clinical disease severity, age, disease aetiology and the presence of cachexia.

Our findings differ from the results of Katz and colleagues[10] who demonstrated that CHF patients with higher TNFα levels showed higher post-ischaemic blood flows and better blood flow responses to acetylcholine. Those authors[10] found no significant correlation between peak or resting blood flows and TNFα levels in the 10 heart failure patients with detectable amounts of TNF. There are important differences between their study and ours. Katz *et al.*[10] had no patients with ischaemic heart disease (here 19/34), none in functional NYHA class IV (here 4/34), and none had sodium levels <136 mmol/l (here 9/34). Our heart failure patients were slightly older (63 ± 11 vs. 58 ± 11, mean $\pm$ SD) and we included cardiac cachectic patients (not specified by Katz *et al.*[10]). Differences between the arm and leg circulations in CHF potentially exist, and they may partly explain the findings. Another potentially important area of difference is the use of different ELISA test kits with different sensitivities (20 pg/ml vs. 3 pg/ml). The TNFα concentrations reported by Katz *et al.*[10] were higher than in our study, but they may not be directly comparable. If the concentrations of TNFα found by Katz *et al.*[10] were indeed higher than in our study, activation of the inducible form of NO synthase might potentially only be seen at these higher concentrations. Katz *et al.* measured peak blood flows in the forearm after 5 min ischaemia, whereas we investigated peak leg blood flows in response to exercise plus an additional 5 min ischaemia.

TNFα may amplify agonist-induced NO expression. But TNFα may also blunt blood flow responses to exercising muscle. The latter effects could be of greater importance in cardiac cachexia, when TNFα is chronically elevated[6] and peak leg blood flow becomes the best predictor of exercise capacity.[5] Multiple factors increase or decrease the effects of TNFα, and its actions vary between tissues. For instance, when TNFα levels are elevated in plasma, it may contribute to skeletal muscle cachexia, whereas local TNFα production in the brain causes anorexia.[9] In endothelial cells, TNFα can cause rearrangement of the cytoskeleton, increased permeability to albumin and water, enhanced expression of activation antigens, induction of surface procoagulant activity and IL-1 release.[16] All of these actions could impair endothelial function.

At the molecular level, TNFα increases the expression of inducible NO synthase in vascular endothelial cells,[17] but also reduces constitutive NO synthase mRNA in these cells.[18] TNFα reduces the half-life of NO, and increases superoxide anion release of endothelial cells,[19] the latter being potentially damaging to the endothelium. Additionally, TNFα may affect other cell systems that are important for vasodilation, such as the calcium transport system.[20,21] These conflicting actions, and the fact that tolerance to TNFα action can result from prolonged exposure,[22] may also explain the differing results between our study and that of Katz *et al.*[10] The reasons for these differences are not known.

Limitations

The total amount of TNFα measured in plasma consists of the total amount of free TNFα molecules and the amount of TNFα bound to soluble receptors (TNF-R1 and TNF-R2). Both types of TNFα receptor can affect TNFα bioactivity.[23] Both test kits used measure TNFα, whether receptor-bound or free. We did not directly measure the bioactivity of TNF in the blood samples of these CHF patients. This study does, however, show a close relationship between peak leg blood flow and the plasma concentration of TNFα. This may indicate a significant pathophysiological role for TNFα in CHF, i.e. that raised TNFα contributes to impaired peripheral vasodilation.

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