

Pro-inflammatory cytokine release by peripheral blood mononuclear cells from patients with advanced pancreatic cancer: Relationship to acute phase response and survival

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Received January 8, 2009; Accepted February 11, 2009

DOI: 10.3892/or_00000328

Abstract. The acute phase protein response (APPR) and peripheral blood mononuclear cell-derived inflammatory cytokine production was assessed in patients with advanced pancreatic cancer and age-matched healthy volunteers. We examined the relationship between the APPR, cytokine production and survival in these patients. Forty-two patients with pancreatic cancer cachexia and twelve age-matched healthy controls were recruited. The nutritional status, Karnofsky performance score, C reactive protein (CRP), serum interleukin-6, and *in vitro* monocyte interleukin-1 and interleukin-6 production were measured. The dates of death of the pancreatic cancer patients were subsequently obtained and appropriate patient variables at baseline were entered into a Cox's proportional hazards model. The cancer patients had significantly lower: body mass index, Karnofsky performance score, serum albumin and elevated CRP and stimulated interleukin-6 production. Both univariate and multivariate analysis demonstrated a strong association between tumour stage, CRP, stimulated interleukin-6 production and survival. Monocytes in cachectic pancreatic cancer patients are primed to produce high levels of interleukin-6 when stimulated. Overproduction of interleukin-6 has a negative impact on survival. Decreased survival is associated with an elevated APPR. While the elevated APPR is probably related to locally produced interleukin-6 in the liver, it seems possible that locally and systemically produced interleukin-6 influences survival.

Introduction

There is increasing evidence that systemic inflammation (due in part to pro-inflammatory cytokines) may mediate a number

of the metabolic processes which contribute to cancer cachexia. We have demonstrated previously that a proportion of weight-losing pancreatic cancer patients have an elevated hepatic acute-phase protein response (APPR) (1) which has been shown to correlate with both increased resting energy expenditure and reduced duration of survival (2). Although a variety of factors, including cytokines and endocrine hormones, are known to influence hepatocyte protein metabolism, the principal regulator of the APPR in human hepatocytes is thought to be interleukin-6 (IL-6) (3). In our previous study (1) of patients with pancreatic cancer, however, no correlation was observed between serum concentrations of IL-6 and the presence of an APPR. Further investigation demonstrated a strong correlation between increased elaboration of IL-6 by peripheral blood mononuclear cells (PBMC) of patients with pancreatic cancer and the presence of an elevated APPR (1), thereby suggesting that local rather than systemic, IL-6 levels might be important. The association between increased pro-inflammatory cytokine production, elevation of the acute phase response, hypermetabolism and shortened duration of survival in patients with pancreatic cancer therefore, makes the cytokine-hepatocyte axis a potential target for therapeutic intervention. This potential as a target in cachexia is increased further by the observation that pro-inflammatory cytokines may also contribute to the anorexia frequently observed in this syndrome (4-6).

In this study we examine the relationship between systemically and locally produced cytokines, the acute phase response, weight-loss and survival in a group of cachectic pancreatic cancer patients. Patients with advanced pancreatic cancer were studied as a model of cachexia as it is almost invariably present in this patient group (7). In addition, chemotherapy was not a standard treatment option during the study. Chemotherapy and its potential complications could not therefore act as confounding variables on weight loss, cytokine production, acute phase response or survival.

Patients and methods

Study design. The study was undertaken at one centre (Royal Infirmary of Edinburgh) on patients with advanced pancreatic cancer (n=42). The control group were attending the hospital

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Key words: cachexia, cytokines, C reactive protein, interleukin-6

for elective surgical procedures. At baseline of the study, the weight, height, grip-strength and body composition of the patients were measured. Blood (50 ml) was withdrawn. The blood was separated into serum, plasma and polymorphonuclear cell fraction as described below. Anthropometric measurements and blood samples were taken from each subject. The cancer patients were then followed up in the out-patient setting. The dates of death of patients were subsequently obtained from their primary care physician.

Cancer patients. Forty-two patients with unresectable pancreatic cancer who had been referred to the Royal Infirmary of Edinburgh were included in the study. Patients had lost >5% of their pre-illness stable weight over the previous six months, had a Karnofsky performance score of 60 or more, and had a life expectancy greater than two months. All patients underwent staging by CT scan. Twenty-four patients had histological proof of diagnosis and eighteen patients were diagnosed on the basis of unequivocal clinical (operative) or radiological (CT scan) findings. Patients with pancreatic cancer were selected for this study as these patients usually experience severe weight loss associated with cancer cachexia. Patients were excluded if they had undergone surgery, endoscopic stenting, radiotherapy or chemotherapy during the previous four weeks, had other active medical conditions (e.g. major gastrointestinal disease, chronic renal failure, uncontrolled diabetes, and human immunodeficiency virus), a body mass index (BMI) >30 kg/m² or had received medication which could profoundly modulate metabolism or weight, in particular, the use of fish oil or n-3 fatty acid preparations exceeding 200 mg/day EPA or one capsule of fish oil/day within the previous 90 days. At the time of enrolment no patients had jaundice, pyrexia, severe anaemia, clinical or radiological evidence of infection and none were taking steroids at doses above that for physiological replacement. No patients had ascites or dependent ankle oedema. Pancreatic enzyme supplements were administered if patients had or developed clinical evidence of steatorrhoea. The Lothian Region Ethics Committee for human research approved the protocol, and written informed consent was obtained from all patients and controls. Procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 1983.

Healthy controls. Twelve age-matched healthy controls were recruited. These were patients admitted to the Royal Infirmary of Edinburgh for elective surgery for benign conditions: hernia repairs and gallbladder removal. All controls were weight stable. The same exclusion criteria were employed as those for the cachectic cancer patients.

Weight and body composition. At the initial assessment, pre-illness stable weight and duration of weight-loss were self reported. Patients height was measured and they were weighed on spring balance scales (Tanita Solar Powered Scale Model 1618, Tanita, Uxbridge, Middlesex, UK) without shoes and wearing light clothing.

Body composition was assessed using a Xitron Hydra multiple frequency bioelectrical impedance analyser (Xitron Technologies, San Diego, CA, USA) as previously

described (8). Resistance was measured at 5 and 200 kHz. Total body water was derived using equations validated in a similar patient group (8). Lean body mass (LBM) was calculated assuming that lean tissue contains 73% water.

Physician-assessed physical function score. Patients were assessed for their level of physical function using the Karnofsky performance score.

Serum markers of systemic inflammation. Clotted blood (10 ml) was centrifuged at 1,500 rpm for 30 min and separated serum stored as 1 ml aliquots at -70°C pending batch analysis at the study end. Serum concentrations of IL-6 (Quantikine, R&D Systems, Abingdon, UK), and C reactive protein (CRP) (Dako, High Wycombe, UK) were measured by ELISA. Limits of detection were 0.5 pg/ml and 1 mg/ml respectively. Coefficient of variation for all assays was <9% across the concentration range studied.

Isolation of PBMC. Peripheral blood nuclear cells (PBMC) were separated from 20 ml of heparin anticoagulated blood on a hypaque gradient (Histopaque 1077, Sigma, Poole, UK) by centrifuging at 1,500 rpm for 30 min. Cells from the interface were removed and washed 3 times in cell culture medium [Roswell Park Memorial Institute (RPMI) medium 1640, Life Technologies, Paisley, UK] with penicillin, streptomycin and glutamine (2 mmol/l) (Sigma) added. Cells were resuspended, counted and cultured in 96-well, flat-bottomed tissue culture plates (Costar, Cambridge, MA, USA) at a concentration of 2×10^5 per well in 200 μ l cell culture medium with 10% fetal calf serum with penicillin, streptomycin and glutamine. PBMCs were then cultured in the presence or absence of 10 μ g/ml *E. coli* lipopolysaccharide (Sigma, Poole, UK). Supernatants from PBMC cultures were removed at 24 h and stored at -70°C for subsequent analysis.

Cytokine assays. IL-6 and IL-1 β concentrations from supernatants were measured by ELISA (Quantikine, R&D Systems). Limits of detection were 240 and 195 pg/ml respectively for stimulated cells and 30 and 19.5 pg/ml respectively for unstimulated cells. Coefficient of variation for all assays was <8.8% across the concentration range studied.

Plasma fatty acid analysis. Plasma was collected from the top of the hypaque gradients and stored as 5 ml aliquots at -20°C pending batch analysis at the study end. Analysis of EPA in patient plasma phospholipids before study commencement was performed by gas chromatography as described previously (9).

Statistical analysis. Results are expressed as mean (standard error of the mean: SEM). A Student's unpaired t-test for independent samples was used to look for differences between groups. Categorical variables on an ordinal scale (Karnofsky performance score) were analysed by the Mann-Whitney U test for unpaired samples. Correlations were examined using Pearson's method. Survival analysis was performed using Cox's proportional hazards model. For all tests, significance was considered at the $p < 0.05$ level.

Table I. Baseline characteristics of 42 weight-losing patients with unresectable pancreatic cancer compared with 12 age matched healthy controls.

	Cancer patients (n=42)	Healthy controls (n=12)	p-value
Sex (M:F)	22:20	5:7	0.532
Age (years)	68 (1)	68 (3)	0.809
Stage of disease			
II	19	NA	NA
III	10		
IV	13		
Body mass index (kg m ⁻²)	21 (1)	27 (1)	0.001
Lean body mass (kg)	43 (1)	47 (2)	0.197
% weight loss from usual weight	21 (1.1)	NA	NA
Karnofsky performance score			
100	-	12	0.000
90	5		
80	11		
70	18		
60	8		
Plasma EPA (%)	0.88 (0.09)	0.71 (0.16)	0.361
CRP (mg/l)	22.9 (7.0)	1.7 (0.2)	0.004
Serum albumin (g/l)	38 (0.87)	43 (0.42)	0.004
Serum IL-6 (pg/ml)	18 (6)	5 (2)	0.057
Unstimulated PBMC IL-6 sups (pg/ml)	3100 (700)	4100 (900)	0.410
Stimulated PBMC IL-6 sups (pg/ml)	66200 (6800)	25200 (3300)	0.000
Unstimulated PBMC IL-1 sups (pg/ml)	169 (46)	255 (74)	0.330
Stimulated PBMC IL-1 sups (pg/ml)	3809 (454)	3659 (415)	0.808

Values are mean (SEM) or total number of patients; comparisons by Student's t-test for independent samples, or Mann-Whitney U-test for KPS; sups, supernatants.

Results

The characteristics of 42 weight-losing pancreatic cancer patients at baseline are shown in Table I. The cancer patients were elderly (mean age: 68 yrs) and were malnourished having lost, on average, 21% of their pre-illness stable weight and with a significantly reduced mean body mass index of 21. In general the patients' Karnofsky score was moderately impaired indicating that they were self-caring but unable to carry on normal activities or do active work. There was no difference in plasma EPA levels between cancer and healthy control patients.

The cachectic cancer patients exhibited an acute phase response with an elevated serum CRP and a trend towards elevated serum IL-6 levels (Table I). LPS-stimulated (but not unstimulated) PBMC from patients produced significantly higher levels of IL-6 compared with healthy controls. In contrast, both unstimulated and LPS-stimulated PBMC IL-1

production rates were not significantly different between the cancer patients and healthy controls.

CRP levels inversely correlated with Karnofsky performance score ($r=-0.301$, $p=0.027$) and serum albumin ($r=-0.419$, $p=0.002$). Serum CRP levels in the cancer patients and healthy controls ($n=54$) correlated with PBMC IL-6 production rates in both unstimulated ($r=0.582$, $p=0.000$) and stimulated ($r=0.388$, $p=0.005$) conditions. There was no correlation between serum CRP levels and serum IL-6 ($r=0.014$, $p=0.920$). There was no significant correlation between serum CRP and PBMC IL-1 production rates either in unstimulated ($r=0.223$, $p=0.063$) or LPS-stimulated ($r=0.223$, $p=0.105$) conditions.

Survival analysis was performed on the 42 cancer patients using Cox's proportional hazards model (Table II). Univariate analysis demonstrated a strong association between tumour stage, CRP and LPS-stimulated PBMC IL-6 production and survival. There was a trend between BMI and

Table II. Survival analysis of 42 patients with unresectable pancreatic cancer using Cox's proportional hazards model.

Variable	Univariate analysis	Multivariate analysis
Sex	0.415	
Age	0.664	
Karnofsky performance status	0.180	
Stage	0.005	0.004
Body Mass Index	0.059	0.032
% weight loss from usual weight	0.267	
Serum albumin	0.562	
CRP	0.000	0.000
Serum IL-6	0.226	
Unstimulated PBMC IL-6 sups	0.224	
Stimulated PBMC IL-6 sups	0.000	0.000
Unstimulated PBMC IL-1 sups	0.716	
Stimulated PBMC IL-1 sup	0.176	

sups, supernatants

survival. Each of these factors retained a significant association with survival on multivariate analysis.

Discussion

Often used to describe severe weight loss, the word cachexia really represents a complex, multifactorial syndrome in which some or all of the following symptoms will be exhibited: anorexia, early satiety, hypophagia, fatigue, anaemia, oedema, and weight loss (characterised by excessive lean tissue wasting compared to undernutrition in an otherwise healthy individual). The average weight loss of the cancer patients was consistent with other studies of pancreatic cancer patients at diagnosis (7,10). The significant impact cancer cachexia has on these patients resulted in a highly significant reduction in performance status from normal.

We have been interested in the potential anti-cachectic effects of the n-3 fatty acid eicosapentanoic acid (EPA) (9,10). EPA and fish oils have been shown to reduce *ex vivo* stimulated cytokine production by peripheral blood mononuclear cells from healthy controls (11,12) and patients with pancreatic cancer (13). EPA is present in small quantities in the average diet of our local population. Cancer cachexia may result in taste aversion to certain foods, anorexia and decreased overall calorie intake. We therefore were interested to compare plasma EPA levels in our cancer and control patients in case this might influence serum and *in vitro* PBMC produced cytokine levels. We found plasma EPA levels were the same between our healthy control and cancer patients.

Our results supported previous studies finding an association between cancer cachexia and an elevated acute phase

response (1,14,15). A variety of proinflammatory cytokines, including IL-6 and IL-1 have been implicated in the weight-loss associated with cancer. The precise importance of their involvement remains unclear, but individually they will each induce anorexia, weight loss and an acute-phase protein response (3,16,17). Circulating concentrations of proinflammatory cytokines are difficult to measure and interpret in human health and disease. In cancer cachexia, IL-1 is infrequently detected, whereas IL-6 is more frequently found to be elevated (18,19). Thus in the present study only serum IL-6 concentrations were measured. The trend towards elevated serum levels in the cancer patients would have been expected to reach significance had the cancer or control groups been larger. However, the lack of statistical significance in this sample size suggests that the association between elevated serum IL-6 level and advanced cancer may be relatively weak. It has been suggested that these proinflammatory cytokines act locally, and so measurement of *ex vivo* production may be a better gauge of activity than circulating concentrations (1). In this study, *ex vivo* measurement demonstrated a highly significant elevation in supernatant IL-6 levels in LPS-stimulated PBMCs from cancer patients when compared to healthy controls. Conversely, PBMC supernatant levels of IL-1, while significantly elevated following LPS stimulation, did not differ between cancer patients and healthy controls. It may be that in pancreatic cancer cachexia PBMC production of IL-6 is upregulated while IL-1 production remains relatively normal. Alternatively PBMC IL-1 production could also be upregulated, but under these particular assay conditions the additional IL-1 remains in the PBMCs and is not released into the supernatant fluid.

An acute phase protein response is associated with, and may well be one of the most important, factors determining cancer cachexia (20). Fatigue and loss of muscle mass are hallmarks of cachexia. It was therefore unsurprising that CRP level correlated inversely with performance status. Serum albumin also correlated inversely with CRP levels. Low-serum albumin levels are frequently found in patients with acute inflammatory conditions including pancreatic cancer cachexia although the cause may be multifactorial (21). The principal regulator of the APPR in human hepatocytes is thought to be interleukin-6 (3). The strong correlation between CRP and PBMC-elaborated but not serum IL-6 suggests that local rather than systemic IL-6 may be responsible for hepatic acute phase protein production in cancer cachexia. Similarly, the lack of correlation between PBMC produced IL-1 and CRP suggests that PBMC produced IL-1 may not influence acute phase protein production in cachexia.

Cox's proportional hazards model (Table II) demonstrates advanced tumour stage to be an independent predictor of survival. Serum CRP level has previously been shown to be a highly significant predictor of survival in patients with pancreatic cancer cachexia, independent of tumour stage (2). Our results confirmed this. The production of acute phase proteins by the liver is associated with the mobilisation of peripheral amino acid stores principally from skeletal muscle. If this process is prolonged or excessive, it may lead to significant depletion of body energy and protein reserves and, thus, contribute to early death.

As PBMC produced IL-6 correlated strongly with serum CRP levels, it is unsurprising that high PBMC produced IL-6 levels are also associated with decreased survival. There may be a direct association by IL-6 contributing to the hepatic acute phase response. However, as both serum CRP and PBMC produced IL-6 remained independently associated with survival on multivariate analysis, locally produced IL-6 may have other effects in addition to stimulating the acute phase response which impact on survival.

It has previously been demonstrated *in vivo* that pancreatic cancers have increased expression of IL-6 mRNA (22). The same authors found that PBMC from cachectic pancreatic cancer patients were primed to produce a 14-fold higher level of IL-6 mRNA when stimulated by IL-6 producing pancreatic cancer cell lines compared to PBMC from non-cachectic patients or healthy controls (22). This response was attenuated by anti-IL-6 antibodies. These authors suggested that pancreatic cancers were directly responsible for stimulating PBMC in cachectic patients. Our results suggest that IL-6 produced by stimulated PBMC may directly influence survival in these patients. Further studies are required to elucidate the complex mechanisms underlying the acute phase response, cytokine production and survival in patients with pancreatic cancer cachexia.

Acknowledgements

We thank the patients for their commitment to the study. We also thank our clinical colleagues whose patients participated in the study (Professor O.J. Garden, Mr. K.K. Madhavan and Mr. Rowan Parks).

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