Presence of tumour inhibits the normal post-operative response in arginine and NO production in non-cachectic mice

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ABSTRACT

We have described recently that cancer patients have low plasma arginine concentrations, even without weight loss being present, suggesting that decreased arginine availability may be a specific feature of the presence of tumour. As arginine is important in post-operative repair, we hypothesized that abnormalities in arginine metabolism in cancer lead to an aberrant post-operative response in arginine and NO metabolism. To investigate this, we studied post-operative alterations in arginine and NO production and the acute-phase response in MCA (methylcholanthrene) sarcoma-bearing mice. Controls, mice with small MCA tumours (< 15% of carcass weight) and large MCA tumours (> 15% of carcass weight) were studied, either with or without undergoing laparotomy. The stable isotopes L-[guanidino-15N2-2H2]arginine and L-[ureido-15N]citrulline were used to study whole-body arginine and NO production rates. SAP (serum amyloid P component) concentrations were measured to assess the acute-phase response. Significance was tested using Mann–Whitney U test. In healthy FVB mice, laparotomy significantly increased whole-body arginine production (from 42 ± 3 to 54 ± 3 nmol·10 g−1·min−1 of carcass weight·min−1), NO production (from 1.1 ± 0.1 to 1.4 ± 0.2 nmol·10 g−1·min−1 of carcass weight·min−1) and levels of SAP (from 4 ± 1 to 115 ± 23 ng/ml), whereas in all MCA tumour-bearing mice baseline values of arginine metabolism and SAP concentration were already elevated and the response to laparotomy was absent. In conclusion, MCA tumour-bearing mice had a disturbed post-operative metabolic response, as evidenced by attenuated post-operative arginine and NO production, concomitant with an attenuated acute-phase response. This indicates that altered arginine metabolism may be an important characteristic of the metabolic changes in cancer.

INTRODUCTION

We have described recently [1] that patients with malignant tumours inducing various degrees of metabolic derangements have decreased plasma arginine concentrations, even without weight loss being present, suggesting that decreased arginine availability may be a specific feature of host metabolism in the presence of cancer. Moreover, up to one-third of cancer patients die from cachexia rather than from cancer itself [2], even after surgical removal of the tumour [3], indicating that cancer-related alterations in the metabolism of the host are a very important factor in determining mortality. In addition, cancer patients undergoing surgical treatment have more post-operative complications [4] and higher mortality [5] than patients undergoing surgery for non-malignant
disease. Apparently, the host’s response to surgery in cancer patients is different from the response of healthy subjects undergoing surgery. Since arginine plays a central role in the host response to surgical trauma, both via NO [produced from arginine in the NOS (NO synthase) pathway] and ornithine (produced from arginine in the arginase pathway), arginine deficiency could compromise post-operative recovery of cancer patients.

Over the last two decades, many studies investigating peri-operative metabolic changes in cachectic cancer patients have been conducted. Nevertheless, the mechanism of cancer-related post-operative disturbances remains to be unravelled, which may be due to the complexity of the metabolic abnormalities that are present in the advanced stage where symptoms, such as weight loss or anorexia, have already become obvious. Therefore we chose to investigate early derangements in the post-operative host response in a non-cachectic tumour mouse model.

The primary aim of the present study was to investigate whether the post-operative response in arginine and NO production was different in the presence of a malignant tumour. The secondary aim was to investigate whether tumour-bearing mice undergoing surgery had an impaired post-operative acute-phase response. Thus we studied basal and post-operative response in arginine/NO production in MCA (methylcholanthrene) sarcoma-bearing mice and healthy controls. To monitor the magnitude of the immune response after surgery, the acute-phase response was measured as plasma concentration of the acute-phase reactant SAP (serum amyloid P component) [6], the mouse homologue of CRP (C-reactive protein) in humans.

MATERIALS AND METHODS

Animals
FVB mice were bred at the Central Animal Facilities of Maastricht University. Mice were fed standard laboratory chow (Hope Pharms) and were subjected to a standard 12 h light/dark cycle. Room temperature was maintained at 22 °C. Water was provided ad libitum throughout the experiment. Male mice of 20–23 g were randomized into six groups: (i) control; (ii) control undergoing laparotomy; (iii) small tumour of 5–15 % of carcass weight; (iv) small tumour undergoing laparotomy; (v) large tumour of >15 % of carcass weight; (vi) large tumour undergoing laparotomy. Based on a power calculation using whole-body arginine production as primary outcome parameter, we calculated the group size to be n = 8. To compensate for mortality during the course of the experiment (related to bleeding during catheter placement and cardiac arrest due to anaesthesia), 12 animals were initially included per group. Food intake was measured daily by weighing the amount of chow in the cage and comparing it with the chow weight of the day before. Experiments were approved by the Ethical Committee of Animal Research of Maastricht University.

Tumour model
MCA tumours were initially induced by subcutaneous injection of 1 mg of MCA (Sigma–Aldrich) in mice in our laboratory. These tumours were maintained in vivo by serially transplanting tumour tissue through a 15 gauge needle. The MCA tumour has been widely used as a model for cancer in metabolic studies in mice and rats and has the histological characteristics of malignant sarcoma with locally aggressive growth [7,8]. In our hands, the tumour did not induce anorexia or weight loss. Control mice were sham-implanted.

Surgical trauma
When the tumour reached 5–15 % of carcass weight (small tumour groups) or >15 % (large tumour groups) [9], a midline laparotomy was performed as a model for surgical trauma. Briefly, ketamine/medetomidine anaesthesia, fluid and temperature control were performed as described previously [10]. Under aseptic conditions, a midline incision from the level of the superior iliac spine to the xyphoid process was made. Intestines were moved aside and wrapped in wet gauze. After 5 min, intestines were put back and the abdomen was closed with wound clips (Autoclip; Clay Adams). After surgical trauma, food was taken away from all mice to prevent differences in food intake between the groups. Water was provided ad libitum.

Metabolic measurements
At 24 h after laparotomy, ketamine/medetomidine anaesthesia and fluid and temperature maintenance were performed as described by Hallemeesch et al. [10]. A primed-constant infusion of stable isotopes of l-arginine {1-[guanidino-15N2]-2H2]arginine ([15N22H2]-Arg); 99 % atom percentage excess} and l-citrulline {1-[ureido-15N]citrulline ([15N]Cit); 99 % atom percentage excess} (Mass Trace) was given via the jugular vein (prime, 146 nmol [15N22H2]Arg/10 g of body weight and 44 nmol [15N]Cit/10 g of body weight; infusion, 960 nmol · 10 g−1 of body weight · h−1 [15N22H2]Arg and 90 nmol · 10 g−1 of body weight · h−1 [15N]Cit). This stable isotope protocol yields tracer/tracee steady state in plasma within 30 min. Blood was collected from the carotid artery and processed as described previously [10]. Amino acid concentrations and TTRs (tracer/tracee ratios) were determined in plasma using HPLC and LC–MS (liquid chromatography–MS), as described by van Eijk et al. [11,12].

SAP ELISA
SAP concentrations in plasma were measured using a sandwich ELISA as described previously [13].
RESULTS

Drop-out

As a consequence of drop-out due to intra-operative mortality, the final group sizes were: (i) control (n = 12); (ii) control undergoing laparotomy (n = 12); (iii) small tumour of 5–15 % of carcass weight (n = 9); (iv) small tumour undergoing laparotomy (n = 9); (v) large tumour of >15 % of carcass weight (n = 7); and (vi) large tumour undergoing laparotomy (n = 7).

Normal post-operative response

In healthy mice, arginine and NO production increased post-operatively by 29 and 27 % respectively (Figure 1), concomitant with a 30-fold increase in SAP concentrations (Table 1). Laparotomy did not affect total amino acid concentrations, including arginine (Table 1).

Effects of tumour

There were no significant differences between tumour-bearing and control mice in carcass weight (control, 25.4 ± 0.4 g; small tumour, 25.5 ± 0.7 g; large tumour, 23.9 ± 0.8 g) or food intake (control, 4.2 ± 0.1 g/day; small tumour, 4.1 ± 0.2 g/day; large tumour, 4.3 ± 0.2 g/day). Compared with controls, plasma arginine concentrations were specifically decreased in mice with small tumours without changes in total amino acid concentrations, including glutamine and citrulline (Table 1). Concomitantly, plasma ornithine concentrations were increased (Table 1). In mice with small tumours, whole-body arginine and NO production were 88 and 54 % higher respectively, than in controls (P < 0.05; Figures 1 and 2), without changes in plasma ornithine concentrations (Table 1). The presence of a small tumour increased SAP concentrations 80-fold, whereas in the presence of a large tumour SAP concentrations rose 280-fold compared with controls (Table 1).

Table 1 Arterial plasma amino acid and SAP concentrations

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LA ST</th>
<th>ST + LA</th>
<th>LT</th>
<th>LT + LA</th>
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</thead>
<tbody>
<tr>
<td>Arginine (µmol/l)</td>
<td>117 ± 8</td>
<td>114 ± 5</td>
<td>90 ± 10#</td>
<td>77 ± 9</td>
<td>147 ± 35</td>
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<tr>
<td>Glutamine (µmol/l)</td>
<td>408 ± 10</td>
<td>385 ± 16</td>
<td>444 ± 26</td>
<td>459 ± 22</td>
<td>435 ± 19</td>
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<tr>
<td>Citrulline (µmol/l)</td>
<td>88 ± 4</td>
<td>78 ± 3</td>
<td>77 ± 6</td>
<td>70 ± 5</td>
<td>86 ± 5</td>
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<tr>
<td>Ornithine (µmol/l)</td>
<td>83 ± 8</td>
<td>85 ± 5</td>
<td>139 ± 13#</td>
<td>120 ± 13</td>
<td>134 ± 11#</td>
</tr>
<tr>
<td>SUMAA (µmol/l)</td>
<td>3128 ± 143</td>
<td>2861 ± 90</td>
<td>3755 ± 277#</td>
<td>3271 ± 221</td>
<td>4295 ± 144#</td>
</tr>
<tr>
<td>SAP (ng/ml)</td>
<td>4 ± 1</td>
<td>115 ± 23#</td>
<td>330 ± 87#</td>
<td>510 ± 124</td>
<td>1116 ± 136#</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M. *P < 0.05 for a laparotomy effect and #P < 0.05 for a tumour effect, as determined using a Mann–Whitney U test. LA, laparotomy; ST, small tumour of 5–15 % of carcass weight; LT, large tumour of >15 % of carcass weight; SUMAA, sum of the amino acids glutamic acid, asparagine, serine, glutamine, glycine, threonine, histidine, citrulline, alanine, arginine, taurine, tyrosine, valine, methionine, isoleucine, phenylalanine, tryptophan, leucine, ornithine and lysine.

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The present study shows that the post-operative increase in arginine and NO production and the acute-phase response that were observed in healthy control mice were absent in MCA tumour-bearing mice. This indicates that the presence of the tumour compromised post-operative arginine metabolism and attenuated the host inflammatory response to surgical trauma.

Arginine is a non-essential amino acid that can be metabolized by NOS or arginase. Conversion by NOS produces NO and citrulline, whereas conversion by arginase yields ornithine. Various malignant human tumours, including lung, colon, prostate and breast carcinoma, contain large quantities of arginase [16–18]. Previously, arginase activity in cancer was shown to be a mechanism for tumour-induced immune suppression via depletion of arginine in the microenvironment of murine lung tumours [19], allowing the tumour to escape the immune response of the host. Subsequently, this mechanism was confirmed in patients with renal cell carcinomas [20]. This may imply that maintenance amounts of arginine in the tumour environment could play a role in host defence against the tumour. The present study demonstrates that the presence of tumour also induces changes in arginine metabolism at the whole-body level, which appeared different in mice bearing small and large tumours. In the first group, plasma arginine concentrations were decreased without significantly affecting whole-body arginine production. In mice bearing large tumours, whole-body arginine production was increased, with slightly (but not significantly) raised plasma arginine concentrations. It can be calculated that arginine clearance (the amount of plasma that is completely cleared of arginine each min [21]) was increased in the presence of both small and large tumours.

It is important at this stage to address the relevance of these observations for human cancers. MCA rodent tumour models have the histological appearance of sarcomas and induce several metabolic characteristics of human malignancies, with wasting of fat tissue [8] and altered protein turnover [22]. In analogy with common human malignancies, such as colonic carcinoma and breast carcinoma, MCA tumours do not induce weight loss. MCA tumour-bearing mice may therefore represent cancer patients in whom no weight loss is present. If so, this may imply that cancer at a phase where no cachexia is present already induces disturbances in host amino acid metabolism. Because human tumours are usually small compared with the patient’s body weight, the experimental model with the small tumour might be the most clinically relevant of the two.

A common treatment for cancer is surgical removal of the tumour. In the post-operative period, several immune processes rely on arginine as a substrate. NO is required for the cytotoxic effect of macrophages, both in animals [23] and humans [24]. Furthermore, arginine is necessary for a functional T-cell response after trauma in mice [25] and humans [26]. Besides, both arginine and its products NO and ornithine are indispensable for wound healing in mice [27]. Recently, NO was shown to enhance collagen synthesis in human tendon cells [28]. Therefore disturbances in arginine metabolism induced by a tumour could have consequences for the host-repair response of cancer patients to surgical trauma. We thus investigated the post-operative response of arginine and NO metabolism in relation to the acute-phase response in tumour-bearing mice.

In the present study, we demonstrate that the presence of the MCA tumour inhibited a post-operative increase in arginine and NO production. If we assume the response of control mice to be normal, this indicates that tumour-bearing mice had a defective post-operative inflammatory response to surgical trauma. Whole-body NO production was measured by calculating the \( \frac{^{15}N_2}{^{15}H_2} \text{Arg to } ^{15}N \text{Cit flux} \). *P < 0.05 for a laparotomy effect, as determined using a Mann–Whitney U test.

**DISCUSSION**

Attenuated post-operative response in tumour-bearing mice

In contrast with controls, laparotomy did not increase whole-body arginine and NO production in the presence of a small or a large tumour (Figures 1 and 2). In mice with large tumours, whole-body NO production decreased 24% after laparotomy (Figure 2). In contrast with healthy mice undergoing surgery, the post-operative SAP concentration did not increase in the presence of a small tumour or a large tumour (Table 1). Similar to controls, laparotomy did not affect total amino acid concentrations, including arginine, in the presence of small tumours. Unlike in healthy animals, laparotomy decreased total amino acid concentrations 25% in mice with large tumours (Table 1).

**Figure 2** NO production

Values are means ± S.E.M. Open bars, without laparotomy; closed bars, with laparotomy. Whole-body NO production was measured by calculating the \( \frac{^{15}N_2}{^{15}H_2} \text{Arg to } ^{15}N \text{Cit flux} \). *P < 0.05 for a laparotomy effect, as determined using a Mann–Whitney U test.

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**Table 1**

<table>
<thead>
<tr>
<th>Group</th>
<th>Small Tumour</th>
<th>Large Tumour</th>
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<td>Baseline</td>
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when metabolism is challenged during metabolic stress. Concomitantly, SAP concentrations did not increase further. In a previous study [29], an attenuated CRP response was also reported in undernourished cancer patients who underwent surgery. However, at that time, no metabolic information was present to explain this phenomenon. Some years later, we reported an impaired metabolic response to surgery in tumour-bearing rats in muscle [9] and intestine [30]. This suggested that the attenuated response to surgery as observed in cancer was not related to malnutrition, but due rather to the presence of the tumour. The present study characterizes further the metabolic insufficiency in cancer, and describes an impaired capacity to mobilize arginine and NO after surgical stress with concomitant suppression of the acute-phase response. Unfortunately, in the MCA tumour model, tumour removal is not possible without mortality, at least not in our hands, probably due to the relatively large size (approx. 10 mm × 20 mm) of the tumour. Strictly, the currently used model is thus comparable with patients in whom radical resection of the tumour was not possible.

Strikingly, there was a ‘chronic’ acute-phase response ongoing in the presence of the tumour. This is probably different from the ‘acute’ acute-phase response that usually occurs after surgical trauma. We speculate that the ongoing acute-phase response as observed in tumour-bearing mice attenuates the initiation of the inflammatory response upon acute metabolic stress, such as surgical trauma. Hypothetically, this may be due to exhaustion of endogenous sources (possibly amino acids from muscle protein) for substrate for the acute-phase response (possibly acute-phase protein). Because absolute SAP concentrations in tumour-bearing mice were higher than post-operative values in healthy animals, a point for discussion remains whether absolute values or relative increases are important for post-operative recovery. Further studies are needed to resolve this issue.

In conclusion, our results suggest that the acute-phase response normally observed after surgery does not occur in the presence of cancer. This non-responsiveness was concomitant with the absence of an increase in whole-body arginine and NO production after surgery, indicating a role for altered arginine metabolism in cancer. Hypothetically, such changes may contribute to post-operative morbidity and mortality and, if similar changes occur in humans with cancer undergoing surgery, this could have consequences for clinical practice.

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