Stimulation of human breast cancers by dietary L-arginine

Kenneth G. M. PARK1,2, Steven D. HEYS1, Karen BLESSING3, Peter KELLY3, Margaret A. McNURLAN1, Oleg EREMIN1 and Peter J. GARLICK1

Departments of 1Surgery and 3Pathology, Aberdeen University, Foresterhill, Aberdeen, U.K., and 2Rowett Research Institute, Bucksburn, Aberdeen, U.K.

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1. The amino acid L-arginine has been shown to enhance immune mechanisms and inhibit tumour growth in experimental animals, but although many of the immunological effects of arginine have been reproduced in man there have been few studies of its effects on human tumours. In this study the effects of arginine on human breast cancers were determined by measuring tumour protein synthesis and comparing this with immunohistochemical assessments of cell proliferation.

2. Patients with breast cancer were randomized to receive either a standard diet or arginine supplementation. At the time of surgery, the rate of tumour protein synthesis was measured by the incorporation of the stable isotope [1-13C]leucine into tumour protein. Tumours were also assessed histologically and by staining for the presence of the activation antigen Ki67.

3. The median rate of tumour protein synthesis was 10%/day (range 5.5–15.8%/day) in the control patients and 25.6%/day (range 9–37%/day) in the patients receiving arginine supplements (P<0.005, Wilcoxon rank sum test). The rates of protein synthesis correlated with Ki67 expression within these tumours (r=0.78, P<0.001). A double-staining technique confirmed that tumour cells, rather than tumour-infiltrating lymphoreticular cells, expressed Ki67.

4. This study demonstrates that, in contrast to animal studies, L-arginine stimulates human tumours in vivo. This represents the first direct evidence that a single amino acid can modulate the behaviour of a human cancer.

INTRODUCTION

Large doses of the amino acid L-arginine inhibit tumour growth and diminish metastatic spread in a number of animal models [1–3]. These effects are thought to be separate from any nutritional effects of arginine, and are possibly mediated through the enhancement of the anti-tumour defences of the host, as has been demonstrated in animals [3]. In man, arginine has similarly been shown to enhance a number of immunological mechanisms. In particular, it increases the rate of lymphocyte transformation with polyclonal mitogens [4–6] and recently we have shown that oral arginine enhances human natural killer and lymphokine activated killer cell activity [7]. These killer cells are believed to play an important role in the host anti-tumour defences [8]. Arginine supplementation, therefore, would appear to be a possible means of beneficially modulating the tumour/host relationship. However, despite a large amount of experimental data from animals, there have been few studies, to date, of the direct effects of arginine on human tumours. There is, however, difficulty in accurately measuring the growth of human cancers in vivo in response to treatment. Conventional static assessment of tumour bulk is insufficiently sensitive to detect differences in response to relatively short periods of treatment. In the present study, therefore, the effect of 3 days of oral arginine on human breast cancers was determined by measuring the rate of tumour protein synthesis in vivo with the stable isotopic label [1-13C]leucine. Rates of tumour protein synthesis have also been compared by using more conventional histological assessments of tumour proliferation.

METHODS

Patients

Twenty patients, admitted to Aberdeen Royal Infirmary for treatment of primary breast cancer, were recruited into the study. Patients gave their written informed consent for participation in the study, which had been approved by the Joint Ethical Committee of Aberdeen University and Grampian Health Board. The patients were randomized into two groups; both received a standard hospital diet but, in addition, one group (n=10) was given supplemental arginine (30 g/day) for 3 days before surgery. The arginine was given orally in four divided doses as arginine base (Forum Ltd, Redhill, Surrey, U.K.). All oral intake ceased at 10.00 hours on the evening before surgery and, at the time of surgery, the rate of tumour protein synthesis was determined by incorporation of [1-13C]leucine as previously described [9, 10].

Key words: L-arginine, breast cancer, Ki67 antibody, protein synthesis.

Abbreviations: ER, oestrogen receptor status; NST, no special type.

Correspondence: Dr K. G. M. Park, Department of Surgery, Aberdeen University, Foresterhill, Aberdeen AB9 2ZD, U.K.
Analytical methods

The isotopic enrichment of free leucine in the plasma was determined by gas chromatography-mass spectrometry (GC-MS) on a VG 12-250 quadruple mass spectrometer (VG Masslab, Manchester, U.K.). Preparation of plasma samples for isotope measurements has been described previously [11], and leucine was measured as the tertiary butyldimethylsilyl derivative [11]. Free leucine enrichment in the tumour biopsies was measured by GC-MS using the N-heptfluorobutyryl n-butyl ester derivative under negative chemical ionization [9, 12].

Free and protein-bound leucine in the tumour samples were separated by pulverizing the frozen sample between cooled aluminium blocks and precipitating the protein in perchloric acid. After several washes the protein fraction was hydrolysed in concentrated hydrochloric acid for 24 h at 110°C, and the leucine was separated by ion-exchange chromatography [9]. The 13C enrichment of the leucine was determined by gas isotope ratio mass spectrometry (SIRA 12; VG Isogas).

Calculations

The fractional rate of protein synthesis was calculated from the previously described formula [9]:

\[ k_s = \left( P(t) - P(0) \right) \times 100 / A \]

where \( P(t) - P(0) \) is the increase in tumour leucine enrichment (atom% excess) over the time of incorporation (t, days) and \( A \) is the area under the curve for precursor enrichment (atom% excess \times time). To avoid taking two biopsies of the tumour, before and after leucine incorporation, the baseline enrichment \( P(0) \) was determined from measurements on the plasma proteins [13]. The results are expressed as fractional rates of protein synthesis \( k_s \), i.e. the percentage of the tumour protein synthesized per day.

Protein synthesis

An initial 10 ml blood sample was taken to measure the baseline 13C enrichment of leucine in plasma proteins. Thereafter, [1-13C]leucine [4.0 g/70 kg body weight, 20 atom% enriched in 200 ml of 0.9% (w/v) NaCl (saline)] was injected intravenously over a 10 min period. At approximately 45 min after the start of the leucine injection, anaesthesia was induced with propofol (ICI Pharmaceuticals, Alderley Park, Macclesfield, Cheshire, U.K.) and was maintained with nitrous oxide and isoflurane (Abbott Laboratories Ltd, Wokingham, Berks, U.K.). Venous blood samples were taken at regular intervals up to 90 min after injection of [1-13C]leucine. The tumour was removed by lumpectomy (approximately 60–90 min after the start of the leucine injection) and representative samples were immediately frozen in liquid nitrogen. These were subsequently stored at −70°C until subjected to analysis.

Tissue histology

In all cases, paraffin sections were cut and stained with haematoxylin and eosin. Assessments were made of the histological type of the tumour, mitotic rate and oestrogen receptor status (ER).

In cases where the biopsies were large enough, cryostat sections were cut from frozen tumour samples and were labelled with Ki67 monoclonal antibody (Dako Ltd, Amersham, Bucks, U.K.). The antibody was then visualized by the streptavidin–biotin technique, in which the immunoreactive sites are stained brown after reaction of the streptavidin–biotinylated complex (Dako Ltd) with 3,3'-diaminobenzidine tetrahydrochloride (Sigma, Poole, Dorset, U.K.) [14]. A cell was considered Ki67-positive if there was obvious nuclear staining, and the percentage of tumour cells staining with Ki67 was assessed as the mean of 10 high-power field examinations by one pathologist, who was unaware of the treatment group. In a further two cases, in which the patients had been given arginine, cryostat sections were labelled with Ki67 and either a mouse anti-human macrophage monoclonal antibody (M718; Dako) or an anti-CD2 monoclonal antibody (M720; Dako), CD2 being expressed on T lymphocytes within the tumour. Ki67 labelling was visualized with a streptavidin–biotin technique and the second antibody with an alkaline phosphatase anti-alkaline phosphatase

### Table 1. Details of the tumours in control patients (n=10) and arginine-fed patients (n=10). Tumours were staged according to the TNM system of the UICC. Abbreviations: IS+, large in situ component; LOB, lobular carcinoma; COM, comedo carcinoma; –, not done.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Clinical stage</th>
<th>Histological type</th>
<th>Ki67 staining (%)</th>
<th>Protein synthesis rate (%/day)</th>
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<td></td>
</tr>
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<td>LOB</td>
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(APAAP) technique [15]. In all cases, cell counting was performed with an image analyser, and the results for each patient were expressed as the mean of 10 randomly selected high-power field examinations.

Additional analytical techniques

Plasma amino acid profiles were measured in both groups of patients with a Chromospek automated amino acid analyser [16]. Plasma insulin levels were determined by using a radioimmunoassay technique [17].

Statistics

Results are expressed as medians (range), and the significance of differences between groups was assessed by the Wilcoxon rank sum test. Correlations between the rate of tumour protein synthesis and histological indices were assessed by using Spearman's rank correlation.

RESULTS

There was no significant difference in the ages of the patients between the two study groups. At the time of study, all patients were free from overt metastatic disease. However, one patient in the arginine-fed group was subsequently found to have both liver and skeletal metastases.

Most patients found the arginine 'bitter' to taste, but compliance was good, as judged by the weight of arginine remaining each day. Two patients developed mild diarrhoea, but in both cases this settled rapidly after the arginine was discontinued. Details of the individual tumours are presented in Table 1. Histologically, most tumours were invasive ductal carcinomas of no special type (NST). Two patients in the arginine-supplemented group, however, had tumours with large in situ components, one of these also having a comedo carcinoma. An additional patient in the arginine-supplemented group had an invasive lobular cancer and one patient in the control group had a lobular component to her tumour.

The median plasma arginine concentration at the time of biopsy in the patients given arginine supplements was 230 μmol/l (range 198–306 μmol/l) compared with 160 μmol/l (range 132–208 μmol/l) in the control patients. The plasma insulin levels were similar in the two groups, ranging from 0 to 35 μ-units/ml (median 12 μ-units/ml) in the arginine-treated group and from 0 to 29 μ-units/ml (median 15 μ-units/ml) in the control group.

After injection of [1-13C]leucine to measure tumour protein synthesis rates, there was a linear fall in plasma leucine enrichment in each individual. The free leucine enrichment in the tumours at the time the biopsy was taken varied between 88 and 96% (median 94%) of the enrichment of plasma leucine. The plasma free leucine enrichment therefore was used as a measure of the precursor for protein synthesis in the calculation of rates of protein synthesis. The median rate of protein synthesis in the arginine-supplemented tumours was 25.6%/day; this was more than twice the median rate in the control group (10.0%/day; P<0.005, Table 1). The two patients with the high carcinoma in situ components in their tumours had the lowest rates of protein synthesis in the arginine-supplemented group, but the lobular carcinomas had rates of protein synthesis comparable with the other tumours. If statistical comparisons are made excluding these four (i.e. using only tumours of NST) there is still a statistically significant difference between the groups (P<0.01).

There was no difference between the groups in terms of mitotic rate, ER status or cellularity of the tumours. Expression of the activation antigen Ki67 was significantly higher in the tumours of the arginine-supplemented patients compared with those of the control patients (P<0.01, Table 1). Fig. 1 shows the correlation between the rates of tumour protein synthesis and the expression of Ki67. The correlation coefficient was 0.78 (P<0.001). However, despite the high correlation coefficient, one tumour was distant from the other points on the scatter plot (Fig. 1) with a rate of synthesis of 9.0%/day and a Ki67 expression of 62%. This patient was one of the two who had a prominent carcinoma in situ component within her tumour and therefore was not typical of the group as a whole. There was insufficient tissue from the other carcinoma in situ tumour to allow assessment of Ki67 expression.

Double staining with lymphoreticular cells, in addition to Ki67, showed Ki67 expression to be present predominantly on the tumour cells. Of 2591 cells counted in 10 high-power fields from one tumour, 12% of the cells (cytologically tumour cells) expressed Ki67; 20% expressed the lymphocyte marker (CD2) and 50% were macrophages. However, only three macrophages and two CD2-positive lymphocytes co-expressed Ki67. In the second tumour, with double staining, 26% of the cells expressed Ki67 (cytologically tumour cells), 24% were lymphocytes (CD2-positive) and 30% were macrophages; only 2% of the cells expressing the Ki67 antigen were macrophages or CD2-positive lymphocytes.

DISCUSSION

The data from this study show that large amounts of oral arginine can stimulate protein synthesis in the tumours of patients with breast cancer, the rate of synthesis in the arginine-supplemented patients being more than double that of the control group. There was also a marked stimulation in the expression of the activation antigen Ki67 in the tumours of the arginine-supplemented patients.

It has previously been demonstrated that changes in rates of protein synthesis are the main determinants of tumour growth in vivo [10, 18, 19]. Protein synthesis has also been used as an index of change in tumour growth in vivo [10, 20, 21], but there was no correlation between rates of tumour protein synthesis and the mitotic rate of the tumours in this study. However, previous authors have emphasized the difficulties in interpreting mitotic rates in paraffin-embedded tissues and that these frequently represent an underestimate of the true rate of tumour cell proliferation [22, 23]. That the stimulation in protein...
synthesis does represent an increase in tumour cell proliferation was indicated by the close correlation between the rates of tumour protein synthesis and the expression of the activation antigen Ki67. Furthermore, double-staining studies confirmed that Ki67 expression was predominantly on the tumour cells themselves. Expression of this antigen is known to occur when cells enter the cell cycle from the resting G₀ phase; Ki67 expression, therefore, is thought to correlate with the fraction of cells within a tumour in the growth phase [24]. It is thus probable that the present findings represent a stimulation of tumour growth by L-arginine supplementation. A possible exception may be carcinomas in situ, in that the two such tumours in arginine-supplemented patients had low rates of protein synthesis despite one having a high expression of Ki67. However, it is uncertain as to whether these tumours had failed to respond to arginine or inherently had very low rates of protein synthesis.

The findings of the present study are in marked contrast with much of the published animal data, in which arginine was found to be inhibitory, reducing tumour implantation, growth and metastatic spread [1-3]. Animal tumours, however, frequently differ from human cancers in their mode of induction, method of establishment and immunogenicity. Many animal tumours are highly immunogenic [25], and the observed effects of arginine on tumour growth in animals may simply reflect a predominance of the known immune stimulatory effects over any direct effects on the tumour [4-7]. Indeed, arginine has been demonstrated to enhance the growth rate of a poorly immunogenic mouse tumour [2].

There have been very few studies to date of the effects of arginine on human tumours. Cho-Chung et al. [26] found that dibutyryl arginine reduced the growth of a human breast cancer cell line in vitro. The concentration used in these experiments (6 mmol/ml) was far in excess of the plasma arginine concentrations obtained in vivo in our study, even after 30 g of arginine/day. Our own studies in vitro have confirmed an inhibition of human tumour cell growth at these high doses of arginine, but at lower doses we were able to demonstrate an enhancement of protein synthesis and growth [27]. Furthermore, in athymic nude mice (lacking competent cellular immunity) arginine supplementation enhanced both the rate of growth and protein synthesis of a xenografted human lung tumour [27].

The mechanism of this stimulation of tumour protein synthesis is not clear. There was no obvious relationship between the plasma arginine concentration and the rate of tumour protein synthesis or Ki67 expression, but this may simply reflect the tracing of measurements, i.e. 12 h after the last dose of arginine. Arginine is a potent secretagogue for a large number of hormones [3]. However, when given in divided doses (as in the present study and our previous studies [7]), we have found no difference in the plasma insulin level, which can be used as a measure of arginine-induced endocrine stimulation. There has been much recent interest in the metabolism of arginine via the deiminase pathway to produce nitric oxide [28, 29]. Nitric oxide has been shown to influence the function of a number of different cell types by a modification of the cyclic AMP/cyclic GMP ratio [30]. Although the deiminase pathway results in an inhibition of experimental tumours, its effect on human tumours is uncertain, and the resultant alterations in free-radical levels may actually enhance tumour growth [31]. Arginine is also a precursor, via ornithine, of polyamines, low-M₁ compounds known to be required for increased cell turnover [32]. Paradoxically, however, large doses of arginine inhibit the key regulatory enzyme, ornithine decarboxylase, in polyamine synthesis [31].

Further investigation is obviously required to determine the underlying mechanism for the effects of arginine on tumours, but this study has shown for the first time that the behaviour of a human tumour, in vivo, can be modified by a single amino acid. This provides a potentially important means of therapeutically modulating tumours, not by inhibiting growth (as suggested by some of the animal studies), but by stimulating tumour cell turnover and, therefore, sensitizing these cancers to cell-cycle-specific chemotherapeutic agents [33].

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