Tumour cell growth in culture: dependence on arginine

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ABSTRACT

The amino acid arginine has been shown to affect the growth of several tumours, although the mechanisms of its action are not clear. In the present study, using a human breast tumour cell line (MCF-7), we investigated the arginine requirements of tumour cells for optimal protein synthesis and growth, and the metabolic pathway responsible for the arginine-dependent growth. The results showed that MCF-7 cells are highly dependent on arginine for growth and that the requirement for arginine is much higher than for an indispensable amino acid, leucine, indicating that arginine is needed for pathways other than protein synthesis. In arginine-free cultures, growth could be completely restored by the urea cycle intermediate citrulline. However, arginine could not be replaced by the urea cycle intermediate and the direct precursor for polyamine synthesis, ornithine, or by the polyamine putrescine, suggesting that the high dependence on arginine is not due to a requirement for polyamine synthesis. Moreover, inhibition of NOS [NO (nitric oxide) synthase] did not affect cell protein synthesis and growth, and the arginine analogue and substrate for NOS, homoarginine, could not replace arginine, implying that the conversion of arginine into NO is not involved in the growth-promoting effects of arginine. The major determinant for the high dependence of MCF-7 cells for arginine was found to be the irreversible conversion of this amino acid into ornithine by the intracellular enzyme arginase. The conversion into ornithine caused a progressive depletion of arginine from the culture medium, which ultimately inhibited cell protein synthesis and halted growth. Intracellular arginase activity may be the major factor determining the requirement for arginine of all cells in culture.

INTRODUCTION

Tumour cells are particularly dependent on arginine for growth [1,2], although the reasons for this requirement are not completely clear. Dietary supplementation with arginine has been shown to stimulate tumour growth in patients with breast cancer [3] as well as to stimulate the growth of some experimental tumours [4–10], suggesting that this amino acid might have a specific role in promoting and/or regulating the proliferation of tumour cells. Three aspects of arginine metabolism may provide insight into the importance of this amino acid for the regulation of tumour growth. Arginine is primarily needed for protein synthesis. It is also the precursor for both polyamines and NO (nitric oxide) synthesis and these pathways could be directly or indirectly implicated in its effects. The importance of polyamines in cell proliferation is well recognized [11–13]. Polyamine concentrations are high in rapidly growing tissues and a close association exists between the rate of tumour}

Key words: L-arginine, arginase, L-citrulline, human breast tumour cell, protein synthesis, tumour growth.

Abbreviations: FCS, fetal calf serum; FCSd, dialysed FCS; FSR, fractional synthesis rate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; NMMA, N6-monomethyl-L-arginine; NNA, N6-nitro-L-arginine; NO, nitric oxide; NOS, NO synthase.

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growth, the induction of rate-limiting enzyme ornithine decarboxylase for polyamine synthesis from ornithine and polyamine accumulation [11,12,14]. Arginine may, therefore, enhance tumour cell proliferation by increasing polyamine synthesis. Alternatively, synthesis of NO from arginine, which is mediated by the NOS (NO synthase) enzyme family, has also been demonstrated in several tumour cells [15–17] and solid human tumours [18–20]. NO production has been implicated in the promotion and/or regulation of tumour cell growth [21–24] and it may represent another mediator of the effects of arginine.

The experiments described in the present study were designed to investigate the effect of arginine on metabolic activity and growth of tumour cells in culture and determine the mechanism of action. Since human breast tumours have been shown to be stimulated in vivo by arginine [3], a human breast tumour cell line (MCF-7) was chosen for this study. By studying MCF-7 cells in culture, the direct effect of arginine on tumour cell proliferation could be evaluated. The potential mechanisms for arginine stimulation of tumour cell proliferation through protein synthesis, polyamine production and NO formation have been investigated.

Tumour growth was estimated by three different methods. The rate of protein synthesis, measured with \( l-[\text{3H}] \)phenylalanine, was used as an index of cell metabolic activity and as an indirect marker of growth. Cell protein content, which relates to the cell number, was measured in the same assay to confirm that changes in protein synthesis were followed by changes in cell proliferation. Cell growth was also assessed independently by using the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay, which indirectly estimates the number of viable cells in relation to their mitochondrial enzymic activity [25].

**MATERIALS AND METHODS**

**Cell culture**

An MCF-7 human breast tumour cell line, maintained in the Department of Surgery, University of Aberdeen, Aberdeen, U.K., was used throughout the experiments. MCF-7 cells were grown and propagated in a standard complete culture medium [RPMI 1640 culture medium with 2 mmol/l glutamine, 1.2 mmol/l arginine and 2 g/litre bicarbonate (Life Technologies, Paisley, Scotland, U.K.) supplemented with 5 % (v/v) FCS (fetal calf serum; ICN Flow, Thame, Oxfordshire, U.K.), penicillin (50 units/ml), streptomycin (50 mg/ml; Sigma, St. Louis, MO, U.S.A.) and fungizone (2.5 mg/ml; ICN Flow)].

**General protocol**

Prior to study, frozen MCF-7 cells were thawed, subcultured and incubated for 48 h in multi-well plates. After 48 h, the complete medium was replaced with arginine-free RPMI 1640 medium (Life Technologies) supplemented with 5 % (v/v) FCSd (dialysed FCS; 1000 \( M_r \) cut-off; Sigma) and incubated for 15 h, allowing depletion of residual arginine. Experiments were then begun with fresh arginine-free RPMI 1640 medium containing 5 % (v/v) FCSd and appropriate additions. The plates were then incubated at 37 °C, 5 % CO\(_2\) in a humidified atmosphere for various times up to 72 h. Additions included l-arginine, l-leucine (Life Technologies), l-citrulline, l-ornithine, putrescine, l-homoarginine, NMMA (N\(^2\)-monomethyl-l-arginine), NNA (N\(^G\)-nitro-l-arginine) (Sigma), all diluted in arginine- and serum-free RPMI 1640 medium.

**Measurement of cell protein synthesis rate**

Cell protein synthesis rate was measured from the incorporation of \( l-[\text{3H}] \)phenylalanine, with modifications of the method described previously by McNurlan and Clemens [26]. MCF-7 cells were initially plated in triplicate at a density of 1 x 10\(^5\) cells/ml of medium per 9.6 cm\(^2\). On the day of the test, cultures were incubated for 90 min at 37 °C in a 5 % CO\(_2\) incubator with 1 \( \mu \)Ci of \( l-[\text{3H}] \)phenylalanine (Amersham Bioscience, Little Chalfont, Bucks, U.K.) and 2.5 \( \mu \)mol \( l \)-phenylalanine/ml of medium. Plates were then cooled rapidly on iced water, the supernatant was removed, and the cells were gently washed three times with ice-cold PBS. Cells and supernatants were stored frozen at −70 °C until further analysis.

Cell protein was precipitated with ice-cold perchloric acid (20 g/litre), washed several times, solubilized in 0.3M NaOH for 1 h at 37 °C and the radioactivity counted (Packard, 4400 series scintillation counter). Protein concentration was also measured by the method of Lowry et al. [27] adapted for automated analysis.

FSR (fractional rate of protein synthesis) was calculated from the incorporation of \( l-[\text{3H}] \)phenylalanine into the cell protein and the radioactivity of the free amino acid in the culture medium, using the formula [28]

\[
\text{FSR} (\%/\text{day}) = \left( \frac{\text{SA}_p}{\text{SA}_F} \times t \right) \times 100
\]

where \( \text{SA}_p \) is the specific activity of the protein-bound phenylalanine (d.p.m./nmol), \( \text{SA}_F \) is the specific activity of the free phenylalanine in the culture medium (d.p.m./nmol) and \( t \) is the duration of the labelling expressed in days. The incorporation of \( l-[\text{3H}] \)phenylalanine into cell protein was converted into \( \text{SA}_p \) assuming cell protein contained 4 % phenylalanine. \( \text{SA}_F \) was calculated from the ratio of counts (d.p.m.) to free phenylalanine (nmol) in the culture medium (i.e. amount in RPMI 1640 medium + amount added as flooding dose).

The method assumes no conversion of phenylalanine into tyrosine by MCF-7 cells. This assumption was verified experimentally. Incubation of cells with labelled
phenylalanine (0.8 mmol/l) for 24 h produced no detectable levels of labelled tyrosine in the culture medium.

**Cell growth determination**

Cell growth was measured with colorimetric determination of the conversion of MTT (5 mg/ml; Sigma) into MTT formazan crystals by mitochondrial dehydrogenases of viable cells [25]. Formazan crystals were dissolved in 0.1 M HCl in isopropanol and measured at a wavelength of 570 nm with background correction at 690 nm (Dynatec MR5000).

Cells plated at densities of 2.5, 5, 10, 15, 20, 40 × 10^3 cells/200 µl per 0.3 cm² and grown for 24 h indicated a linear relationship between absorbance and cell density (r^2 = 0.994).

**Nitrite assay**

Nitrite concentration in the cell supernatant was measured with the Griess reagent using sodium nitrite (Sigma) as a standard [29].

**Amino acid analysis**

Amino acid concentrations of cell supernatants were measured with an ion-exchange amino acid analyser (Locarte, London, U.K.) with ninhydrin detection. Norleucine and 6-amino-n-caproic acid (Sigma) were used as internal standards.

**Arginase activity**

The intracellular arginase activity was determined from the conversion of arginine into ornithine as described by Colombo and Konarska [30]. Ornithine production was assessed by the Chinard reaction [30] and quantified with ornithine standard curves that were consistently linear (r^2 = 0.999) over the range of ornithine concentrations used (0–0.2 mmol/l).

**Statistics**

All the results are expressed as means ± S.E.M. of separate experiments. In each experiment, samples were run in three to six replicates. The results were analysed by a one-way ANOVA. Where variances in the data were unequal, log transformation of the data was performed prior to analysis. Correlation between different variables was determined by linear regression analysis and statistical significance was defined as P < 0.05.

**RESULTS**

**Arginine requirement for protein synthesis and growth**

In order to estimate arginine requirements for protein synthesis and growth, MCF-7 cells were incubated in medium containing concentrations of arginine from 0–6 mmol/l for 72 h. Measurements were then made at 24, 48 and 72 h.

The necessity of arginine for MCF-7 cells is demonstrated by the 66 % reduction in protein synthesis after only 24 h incubation in arginine-free medium (P < 0.001 compared with 1 mmol/l arginine; Figure 1A). Cells without arginine decreased in number over the 3 days of incubation and the protein content of cells fell (P < 0.001 compared with 1 mmol arginine; Figure 1B). A similar decline in cell growth in cultures without arginine was obtained with the MTT assay based on mitochondrial dehydrogenase activity, where cell growth was 55 % lower than controls after 24 h (P < 0.0001) and 93 % lower than controls after 72 h (P < 0.0001 compared with 1 mmol arginine; Figure 1C).

When cells were incubated in medium containing 0.4 mmol/l arginine or higher, protein FSR was
maintained for 72 h (Figure 1A). However, at arginine concentrations below 0.4 mmol/l, FSR declined by 53 % (P < 0.0001 compared with 1 mmol arginine; Figure 1A). This decrease in FSR was accompanied by a 46 % decline in cell number (P < 0.0001 compared with 1 mmol arginine; Figure 1B). The MTT assay indicated a 25 % decline of mitochondrial function at 0.1 mmol/l arginine (P < 0.01 compared with 1 mmol arginine; Figure 1C).

A depression in FSR was detectable even at 48 h of incubation in medium containing 0.1 mmol/l arginine (Figure 1A), although the decline in cell number was not statistically significant (Figures 1B and 1C). In shorter periods of incubation (i.e. 24 h), 0.1 mmol/l arginine was sufficient to maintain protein FSR (Figure 1A), cell protein content (Figure 1B) and growth (Figure 1C).

Deprivation of arginine did not induce an irreversible decline in cell growth, as restoration of arginine to cells deprived for 2 days resulted in increased growth (results not shown).

**Leucine requirement for protein synthesis and growth**

In order to compare arginine requirement with that of the essential amino acid leucine, the same protocol was repeated with varying concentrations of leucine. As with arginine, incubation in a leucine-free medium for even 24 h resulted in a significant decrease in protein FSR (P < 0.01 compared with 1 mmol leucine; Figure 2A), cell protein content (121 ± 4 and 143 ± 4 µg in 0 compared with 1 mmol/l leucine; P < 0.05) and growth (P < 0.005; Figure 2B). However, unlike arginine, leucine at a concentration of 0.08 mmol/l was sufficient to maintain maximal FSRs and cell growth for the 72 h incubation period (Figure 2). For incubation for 48 h, 0.04 mmol/l leucine was sufficient to sustain maximal FSR and cell growth (Figure 2). For a 24 h incubation period, 0.02 mmol/l leucine was adequate to maintain maximal protein FSR and growth (Figure 2). However, cell number was also comparable with control (1 mmol/l leucine) in cultures with 0.02 mmol/l after 48 h and 0.01 mmol/l after 24 h incubation (Figure 2B).

The higher requirement for arginine compared with leucine was not the result of a greater proportion of arginine in the cell protein relative to leucine. In fact, analysis of the amino acid composition of MCF-7 cell proteins indicated that arginine content was approx. 22 % lower than leucine (arginine/leucine ratio, 0.78). Therefore the higher requirement for arginine over leucine suggested that arginine is needed for a pathway other than protein synthesis.

**Determination of the metabolic pathway responsible for the arginine requirement for protein synthesis and growth**

In order to investigate the metabolic pathways of arginine which gave rise to the high requirement for arginine, cell cultures were incubated for 24 h in media containing a limiting amount of arginine (0.02 mmol/l) and the polyamine putrescine, the immediate precursor for polyamine synthesis l-ornithine, a precursor for NO synthesis (l-homoarginine) and the urea cycle precursors for arginine (l-citrulline and l-ornithine). The supplemented groups were compared with control groups incubated with 1 mmol/l arginine. The addition of l-ornithine (1 mmol/l), putrescine (0.5 mmol/l) or l-homoarginine (1 mmol/l) was unable to replace arginine (Table 1). Although not statistically significant, there was a small increase in protein FSR in cultures incubated with ornithine or putrescine and a limiting amount of arginine compared with those incubated with a limiting amount of arginine alone (Table 1). Although not statistically significant, there was a small increase in protein FSR in cultures incubated with ornithine or putrescine and a limiting amount of arginine compared with those incubated with a limiting amount of arginine alone (Table 1). However, the observed increase in protein FSR did not contribute to a demonstrable enhancement of cell growth as determined by the cell protein content (Table 1). Only l-citrulline (1 mmol/l) was able to completely replace arginine, restoring a maximal protein FSR and cell protein content (Table 1). Comparable results were obtained when cell growth was assessed with MTT assays in cultures incubated for 24 h in arginine-deficient media supplemented with the various metabolites, as shown in Figure 3.
Table 1  Tumour cell protein FSR and cell protein content measured after 24 h of incubation in media containing arginine alone and in combination with different metabolites

Values are means ± S.E.M., n = 6 from two separate experiments. * P < 0.05 compared with 1 mmol/l arginine. There was no significant difference in FSR or protein content among the groups marked "

<table>
<thead>
<tr>
<th>Treatment</th>
<th>FSR (%/day)</th>
<th>Cell protein content (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine (0.02 mmol/l)</td>
<td>28.3 ± 2.4</td>
<td>109 ± 4.3</td>
</tr>
<tr>
<td>+ Ornithine (1 mmol/l)</td>
<td>35.1 ± 1.2</td>
<td>99 ± 4.2</td>
</tr>
<tr>
<td>+ Putrescine (0.5 mmol/l)</td>
<td>34.4 ± 2.4</td>
<td>100 ± 5.7</td>
</tr>
<tr>
<td>+ Homoarginine (1 mmol/l)</td>
<td>31.6 ± 2.7</td>
<td>103 ± 4.7</td>
</tr>
<tr>
<td>+ Citrulline (1 mmol/l)</td>
<td>83.1 ± 5.7</td>
<td>134 ± 7.4</td>
</tr>
<tr>
<td>Arginine (1 mmol/l)</td>
<td>81.3 ± 6.4</td>
<td>144 ± 11</td>
</tr>
</tbody>
</table>

Figure 3  Tumour cell growth estimated with the MTT assay after 24 h incubation in arginine-deficient medium containing various metabolites

Values are mean ± S.E.M. (n = 6). * P < 0.001 compared with cultures treated with 1 mmol/l arginine (control). Arg, arginine; Cit, citrulline; Orn, ornithine; Put, putrescine; Homoarg, homoarginine.

Separate experiments also showed that the addition of putrescine (0.5 mmol/l) to cultures already containing a standard arginine concentration (1 mmol/l) did not affect cell protein FSR and growth after incubation for 24 h (FSR, 78.2 ± 2.5 compared with 81.6 ± 1.1 %/day respectively; n = 3).

Replacement of arginine with citrulline

Cells incubated in medium lacking arginine but containing a wide range of citrulline concentrations (0–3 mmol/l) confirmed that citrulline can entirely substitute arginine for protein synthesis and that the requirements for the two amino acids are similar (Figures 1 and 4). Over the 3-day period, the protein FSRs measured in cultures containing arginine or equimolar citrulline were the same, with the exception of the cultures incubated with 0.1 mmol/l citrulline after 24 h incubation, which showed a lower rate of protein synthesis than those incubated with 0.1 mmol/l arginine (52.9 ± 1.7 compared with 83 ± 7.3 %/day; P < 0.002; Figures 1A and 4A).

Inhibition of NO formation

The effects of NOS inhibitors on tumour cell metabolic activity and growth were determined by incubating cell cultures in medium containing NMMA and NNA. Incubation for 24 h in the presence of 1 mmol/l NMMA or NNA and 0.1 mmol/l arginine did not affect tumour cell protein synthesis (65.25 ± 2.46 and 61.7 ± 2.65 %/day for NMMA and NNA respectively, compared with 65.8 ± 1.90 %/day for arginine alone; n = 5) or cell growth (absorbance, 0.066 ± 0.003 and 0.067 ± 0.007 for NMMA and NNA respectively, compared with 0.077 ± 0.008 for arginine alone; n = 5). Similar results were obtained when measurements were carried out after shorter incubation times (4 and 8 h). When cultures were treated with 5 mmol/l NNA and 0.1 mmol/l arginine for 24 h, although protein synthesis was not affected compared with arginine alone

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(65.5 ± 2.5 compared with 65.8 ± 1.90 %/day; n = 5), but a decrease in cell protein content (83 ± 6.4 compared with 113 ± 11 µg; n = 5; P < 0.05) and cell growth (absorbance, 0.049 ± 0.024 compared with 0.070 ± 0.008; n = 5; P < 0.05) were observed. The inability of arginine even at high levels (10 mmol/l) to reverse this effect (absorbance, 0.045 ± 0.008 for 10 mmol/l arginine + 5 mmol/l NNA compared with 0.070 ± 0.008 for 1 mmol/l arginine; n = 5; P < 0.05) suggested a specific toxic effect of high concentrations of NNA rather than inhibition of NO-stimulated cell growth. No inhibitory effects on growth were observed with 5 mmol/l NMMA (absorbance, 0.080 ± 0.003 for 0.1 mmol/l arginine + 5 mmol/l NMMA compared with 0.080 ± 0.004 for 0.1 mmol/l arginine alone; n = 5).

NO production
Production of NO by MCF-7 cells was assessed by measuring the end product of the arginine/NO pathway, nitrite, in the cell supernatant of cultures grown for 24–72 h at initial arginine concentrations ranging from 0–6 mmol/l. Nitrite concentration was found to be below detectable levels at all of the arginine concentrations over the 3 days.

Amino acid concentrations in the culture medium
The amino acid concentration in the culture medium was measured after 24, 48 and 72 h of incubation in cultures containing arginine or citrulline. In cultures containing arginine, the arginine concentration in the medium decreased with time (Figure 5A). Moreover, the rate of disappearance was approximately the same, regardless of the initial concentration of arginine in the medium. An increase in ornithine was observed corresponding to the disappearance of arginine from the medium (Figure 5B).

Incubation with citrulline at different concentrations did not give rise to detectable arginine in the medium; however, the ornithine concentration increased (Figure 6).

Arginase activity
The intracellular arginase activity was measured after 24, 48 and 72 h of incubation in complete medium. The intracellular arginase activity was 169 ± 8 µmol ornithine/g of cell protein per h on day 1 (n = 3) and significantly decreased to 110 ± 8 µmol ornithine/g of cell protein per h on day 2 (P < 0.01), and to 79 ± 5 µmol ornithine/g of cell protein per h on day 3 (P < 0.03).

DISCUSSION
Arginine is essential for most cells in culture [8,31], although it is not considered an essential amino acid in humans [32]. The present experiments indicate that arginine is essential for MCF-7 human breast tumour cells. Without arginine in the culture medium, protein synthesis and cell growth were depressed (Figure 1). An initial concentration of arginine of 0.4 mmol/l had to be present in the culture medium to sustain maximal growth up to 72 h compared with a requirement of only 0.08 mmol/l for leucine.
Although the importance of arginine in promoting cell growth in culture has been acknowledged for a long time, the mechanisms of its action are still not well known. Arginine functions as an amino acid necessary for the synthesis of protein, which is fundamental for cell survival and proliferation, but arginine also serves as a precursor of other metabolic pathways, including polyamine and NO synthesis, which might mediate cell growth. The growth inhibition observed in the presence of low arginine concentrations might therefore be caused by a block in protein synthesis and/or by a reduction in growth promoters. However, the results indicating that the dependence of cells for arginine in the culture medium was much higher than for leucine, despite the lower arginine content of protein, suggest that the high requirement for arginine does not arise solely from the requirement for protein synthesis.

Arginine is also a precursor for polyamine synthesis. Polyamines are involved in cell proliferation, growth and differentiation of both normal and neoplastic tissue [11,12,14]. However, in MCF-7 cells, the addition of the polyamine putrescine to complete culture medium did not affect cell proliferation and protein synthesis, indicating that, once the minimal requirements are met, increased polyamine production does not itself possess growth-promoting effects. Furthermore, experimental evidence suggested that the depression of polyamine synthesis alone in arginine-deprived cultures does not account for the observed growth inhibition. The addition of putrescine or its direct precursor ornithine did not restore maximal growth in MCF-7 cells with limiting concentrations of arginine (Table 1), in agreement with the observations of Efron et al. [33] that ornithine and putrescine could not replace arginine for mitogen-induced proliferation of lymphocytes. Although a small effect of polyamine on growth cannot be completely ruled out, it would appear that the high requirement of MCF-7 cells for arginine is for the amino acid itself and not for the synthesis of polyamines from arginine.

Although ornithine could not replace arginine for growth and protein synthesis, another urea cycle intermediate, citrulline, was able to completely replace arginine for growth. This implied that MCF-7 cells were able to convert citrulline into arginine, but lacked the intramitochondrial enzyme ornithine transcarbamylase necessary to regenerate arginine from ornithine. As in MCF-7 cells, Tytell and Neuman [2] have shown that ornithine could not substitute for arginine in 12 stable cell lines and 30 primary cultures, whereas the pathway from citrulline to arginine is widely expressed in different cells [34–37].

Arginine could also regulate cell growth through the production of NO. However, in the present study, the NOS substrate, homoarginine, was not able to replace arginine, nor did the inhibition of NOS activity with NNA or NMMA affect tumour cell growth. At very high concentrations of NNA, growth was inhibited. This was more likely to be a specific toxic effect than suppression of NO-mediated cell growth. The effect was non-competitive and was not observed with similar concentrations of NMMA. The reduction in cell growth with 5 mmol/l NNA was not accompanied by a depression in the protein FSR. This observation can be explained by the loss of non-viable cells in the washing procedure. Since only viable cells contribute to the estimate of protein synthesis, the measurement would not be affected by increased cell death. These results, together with the observation that the concentration of the end product of the NO pathway, nitrite, in the culture medium was not detectable, strongly demonstrate that the arginine/NO pathway cannot account for the high requirements of arginine observed in MCF-7 breast tumour cells.

Arginine concentration in the culture medium decreased almost linearly in a time-dependent manner, with declining protein synthesis and growth following the disappearance of arginine from the culture medium (Figure 5A). Parallel with the decrease in arginine, a linear rise in ornithine concentration was also observed (Figure 5B), suggesting that arginine was being metabolized to ornithine. Since ornithine could not be converted into arginine, this process results in an irreversible loss of arginine. In cultures in which citrulline was substituted for arginine, an increase in ornithine concentration was also observed (Figure 6).

The presence of intracellular arginase activity confirmed the mechanism for conversion of arginine into ornithine. Arginase activity relative to cell protein decreased over 3 days of incubation, but the total arginase activity per culture increased due to the increasing cell number. Arginase is found in high concentrations in the liver, but it can be considered a ubiquitous enzyme with lower activities in non-hepatic tissues. Three different isoforms have been described in the rat [38]. Tumours have been reported to have high arginase activity, with mammary tumours in rodents exhibiting much higher arginase activity than the normal mammary tissue [39]. Arginase activity has also been detected in human tumours [40–42]. The role and metabolic function of the extrahepatic arginase is still not clear. In rapidly proliferating tissues, such as tumours, arginase might be important in providing ornithine for polyamine synthesis. However, the results obtained in vitro with MCF-7 cells indicate that ornithine production is far in excess of the demand for polyamine synthesis, questioning whether this is the only physiological role of intracellular arginase. From the present study, it would appear that MCF-7 cells had a high requirement for arginine due to the inability to sufficiently down-regulate arginase activity in the presence of decreasing concentrations of arginine. Although prior experimental growth conditions (i.e. growth in complete medium, followed by incubation
in arginine-free medium for 15 h) may have had some impact on the ability of MCF-7 to regulate arginase activity, the results show that the conversion of arginine into ornithine is not appreciably diminished even when arginine concentration in the medium is low, causing the complete disappearance of arginine from the culture medium (Figure 5). Arginine depletion results ultimately in cell death, although the consequences might not be so dramatic in vivo since arginine would be continuously provided through the blood supply. However, depletion of arginine levels by arginase might be one of the reasons for the observed stimulation of protein synthesis and activation antigen Ki67 expression in breast tumours in women given dietary supplements of arginine [3].

The present study confirmed that protein synthesis rate can be used as a sensitive marker of cell growth in tumour cells. The three methods used to assess tumour cell growth in fact gave comparable results and, overall, a similar response to various treatments. The apparent differences resulted from the different nature of the measurements. The measurement of the cell protein synthesis rate is a dynamic assessment which enables changes in metabolism happening at the time of the measurement to be detected before they result in significant modifications of the cell protein content and number. On the other hand, the assessment of cell growth using the measurement of cell protein content and the MTT assay are based on the number of total or active cells. This explains why, by measuring the rate of protein synthesis, significant changes in cell growth could be detected 12–24 h before they became evident by using the other two techniques.

In conclusion, these experiments show that arginine is an essential nutrient for the growth of MCF-7 human breast tumour cells. They also demonstrate that the requirements for arginine are much higher than for another essential amino acid leucine. Neither ornithine nor the polyamine putrescine could replace arginine for growth, indicating that polyamine synthesis might not be responsible for the growth-promoting effects of arginine. Only the urea cycle intermediate citrulline could completely substitute arginine. The main reason for the high arginine requirement was found to be the irreversible conversion of the amino acid into ornithine by the intracellular arginase, which resulted in arginine deprivation for protein synthesis and a block of cell proliferation. Arginase activity, which is expressed in normal and tumour cells and in a variable amount, may be the main determinant in arginine requirement for all cells in culture.

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