Tumour growth results in changes in placental amino acid transport in the rat: a tumour necrosis factor α -mediated effect

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The implantation of a fast growing tumour (Yoshida AH-130 ascites hepatoma) to late pregnant rats resulted in no changes in fetal growth, this possibly being associated with an important increase in the fetal uptake of maternal-derived amino acids [Carbó, López-Soriano and Argilés (1995) *Endocrinology* **136**, 3579–3584]. The present investigation was undertaken to see whether the presence of the tumour induced changes in placental transport systems. For alanine transport, although no changes in affinity (K_m) were observed, tumour growth resulted in a 192 % increase in V_{max} in the Na^+ -independent component. Kinetic analysis of the Na^+ -dependent component resulted in two clearly different components: while the low-affinity and high-capacity component was unaffected by tumour growth, the high-affinity, low-capacity component of the tumour-bearing rats showed an important increase in V_{max} (78 %). With regard to leucine transport, tumour burden induced important increases in the Na^+ -independent component, not only in K_m (262 %) but also in V_{max} (189 %). Since elevated tumour necrosis factor- α (TNF) concentrations have been reported in this kind of tumour model, we performed the same type of transport experiments in rats

chronically treated with TNF, the results obtained showing great similarities with those observed with tumour growth. The V_{max} of Na^+ -independent alanine transport was also increased by the cytokine (104 %) while no changes were observed in affinity. TNF treatment also induced an increase in the V_{max} (67 %) of the Na^+ -dependent (high-affinity, low-capacity) component while no changes in affinity were observed. Concerning leucine kinetics, TNF treatment, as in the case of tumour growth, also increased K_m (155 %) and V_{max} (72 %) associated with Na^+ -independent transport. Interestingly, treatment with the cytokine increased both the K_m (43 %) and V_{max} (64 %) of the Na^+ -dependent component. The inhibition patterns suggest the existence of more than one Na^+ -dependent transport for alanine although the majority of the amino acid is transported through the A system. The results presented suggest that, during gestation, the mother is able to adapt her placental amino acid transport systems to compensate for the nitrogen drainage associated with tumour growth and thus provide the fetus with enough amino acids to allow its normal growth, and that TNF could be responsible for the triggering of this compensatory mechanism.

INTRODUCTION

Growth involves the accretion of a net quantity of protein, underlining the importance of amino acids as nutrients in fetal life. In this way, the umbilical uptake of amino acids is the primary nutritional supply line for these compounds during fetal life. However, the role of amino acids in fetal metabolism is not restricted to the sole process of tissue protein accretion, since the quantity of amino acids supplied to the fetus exceeds by far the rate of accretion of tissue protein [1,2]. The majority of amino acids (except glutamate and aspartate) are transferred to the fetus in large amounts, where they are oxidized to CO_2 [3]. This is supported by the high rates of urea synthesis present in the fetus of most species [4].

Very few studies have related pathological situations with alterations in amino acid transport to the fetus. A number of studies have attempted to demonstrate an effect of maternal ethanol ingestion upon fetal uptake of amino acids [5]. In spite of the methodological problems involved in this kind of study, the different data suggest an alteration in the placental uptake of amino acids from the maternal circulation during ethanol ingestion. Chronic maternal hyperglycaemia does not seem to have any effects upon alanine transport to the fetus [6]. In the rat, the implantation of a rapidly growing tumour (the Walker 256 carcinosarcoma) in the mother during gestation results in impaired fetal growth [7], but there are no results relating this phenomenon with a decreased fetal amino acid availability caused by tumour burden.

To some extent, the growth and metabolic characteristics of the fetus share important similarities with those of a fast-growing tumour. Both tissues consume appreciable amounts of glucose and amino acids for both oxidation (conversion of glucose into lactate) and protein synthesis involved in growth in the growing fetus and in tumour mass. The control of glucose utilization by the rat fetus is closely related to its insulin levels [6], whereas in the case of most tumours, glucose utilization appears to be unaffected by insulin and is solely dependent on the glucose availability [8]. A similar situation may exist for the utilization of amino acids. Consequently, competition may occur between the fetus and the tumour and the interactions may affect their growth. A previous *in vivo* study has shown that the implantation of a rapidly growing tumour, the AH-130 Yoshida ascites hepatoma, to pregnant rats (the tumour is growing exponentially at the same time as the exponentially growing fetus) resulted paradoxically in no changes in the growing rate of the fetuses [9]. Bearing this in mind, it was our aim to investigate whether the placental amino acid transport systems were affected as a result of tumour growth.

EXPERIMENTAL

Animals

All animals (female Wistar rats) were fed *ad libitum* on a chow diet consisting (by weight) of 54 % carbohydrate, 17 % protein and 5 % fat (the residue was non-digestible material) with free

Abbreviations used: TNF, tumour necrosis factor- α ; MeAIB, methylaminoisobutyrate.

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access to drinking water, and were maintained at an ambient temperature of $22 \pm 2^\circ\text{C}$ with a 12 h-light/12 h-dark cycle (lights on from 08:00 h). They were mated at 60 days of age (180 g body weight approx.). Day 0 of pregnancy was considered to be when the presence of spermatozoa in vaginal smears was detected. 20-day pregnant rats were used in all the experiments.

Biochemicals and radioactive compounds

They were all reagent grade and obtained either from Boehringer Mannheim S.A. (Barcelona, Spain) or from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Radioactive compounds were purchased from Amersham International (Amersham, Bucks., U.K.).

Cytokine administration

Alzet mini-pumps (model 2ML1) with an infusion rate of $10\ \mu\text{l/h}$, were implanted subcutaneously in 13-day pregnant rats (daily dose of murine recombinant $\text{TNF}\alpha/100\ \mu\text{g}$ of protein per kg of body weight). Food intake and body weight were measured daily during the cytokine treatment.

Tumour implantation

A Yoshida AH-130 ascites hepatoma cell suspension (approx. 120×10^6 cells in 2 ml) was injected intraperitoneally, the control rats being injected with 2 ml of 0.9% (w/v) NaCl solution. Yoshida AH-130 is a rapidly growing tumour with a volume doubling time of 1 day. Total cell content was estimated using Trypan Blue staining. All the experiments took place 7 days after tumour transplantation, a day at which the cells are growing exponentially or have reached the stationary phase [10,11]. In order to carry out the experiments with 20-day pregnant rats, they were inoculated previously (day 13) with the tumour.

Placental membrane vesicles

For each vesicle preparation, two or three 20-day pregnant rats were used and their combined placentae (usually 30–40, total weight approx. 20 g) collected in a beaker on ice; all subsequent procedures were carried out at 4°C . Vesicles were prepared as described previously for human [12] and rat placenta [13]. Protein content of homogenate and vesicle fractions was measured by the method of Lowry et al. [14] using BSA as standard. Alkaline phosphatase activity (EC 3.1.3.1) was measured by the method of Vengesa and Hopfer [15].

Transport experiments

Transport experiments were performed at room temperature (range $22\text{--}25^\circ\text{C}$) using [^3H]alanine and [^{14}C]leucine. Aliquots of $20\ \mu\text{l}$ of isolated vesicles suspended in intravesicular buffer (5 mM Hepes, 5 mM Tris, 290 mM sucrose, pH 7.4) were mixed with $20\ \mu\text{l}$ of extravesicular buffer containing 5 mM Hepes, 5 mM Tris and 145 mM sodium or potassium thiocyanate, and either L-[^3H]alanine (ranging from 0.2 to 40 mM) or L-[^{14}C]leucine (ranging from 0.2 to 20 mM), pH 7.4. At the end of the incubation time (5 s, except for the time-course assays), amino acid transport was stopped by the addition of 1 ml of ice-cold stop solution containing 130 mM NaCl, 10 mM Na_2HPO_4 , 4.2 mM KCl, 1.2 mM MgSO_4 and 0.75 mM CaCl_2 , pH 7.4. The full volume was filtered across nitrocellulose filters ($0.45\ \mu\text{m}$ mean pore; Schleicher & Schull, Germany) under vacuum and washed with 5 ml of stop buffer. The filters were set in mini-vials and their radioactivity was determined using a scintillation counter. The non-specific retention of labelled amino acid in the filters was determined using blanks, assayed in the absence of vesicles, and

then subtracted from the total retention data. Transport assays were carried out in duplicate.

Kinetic analysis and statistics

Kinetic analysis (Michaelis–Menten equation) was performed with the ENZ-FITTER program. Statistical comparisons were carried out with Student's *t* test.

RESULTS AND DISCUSSION

Pregnancy is a physiological state characterized by increased food intake and important metabolic changes involving carbohydrate, lipid and protein metabolism. These adaptations are essential to sustain exponential fetal growth at the end of gestation. Especially relevant are the changes associated with increased protein degradation in skeletal muscle [16] and decreased ureogenic capacity of the pregnant rat [17], both allowing for a nitrogen-saving mechanism which is essential to sustain the high nitrogen demand of the growing fetus. Indeed, growth involves the accretion of a net quantity of protein, underlining the importance of amino acids as nutrients in fetal life. In this way, the umbilical uptake of amino acids is the primary nutritional supply line for these compounds during fetal life.

A previous study [9] indicated that the implantation of a rapidly growing tumour during gestation in the rat resulted in no changes in fetal growth in spite of the exponential growth of the tumour. This phenomenon was associated with a decreased maternal total blood amino acid concentration [18] (the levels of leucine, for instance, were 32% lower than in the non-tumour-bearing control group) together with an increased amino acid uptake by the fetus and was not due to an augmentation of placental blood flow [9]. In order to examine the changes in placental amino acid transport systems, we decided to perform transport assays in reconstituted trophoblastic placental membrane vesicles. We chose alanine and leucine in our study for two main reasons. First, our previous *in vivo* study had been carried out using analogues of these amino acids, α -aminoisobutyrate and cycloleucine respectively. Secondly, alanine is considered to be the main gluconeogenic amino acid and leucine is one of the most representative essential amino acids.

Integrity and transport capacity of the placental membrane vesicles

The integrity of the placental membrane vesicles was assessed as previously described [13]. The uptake of L-alanine was linear in the first 10 s and the overshoot was observed at the 45-s interval (results not shown). There were no differences in vesicle volume, as assessed by examining the hyperosmolar behaviour of the vesicles (results not shown).

Tumour growth and amino acid transport

The results obtained (Table 1, Figures 1a and 1b) clearly demonstrate that alanine transport was affected by tumour growth. With regard to the Na^+ -independent component (Table 1, Figure 1a), although no changes in K_m were observed, tumour growth resulted in a 190% increase in V_{max} . Kinetic analysis of the Na^+ -dependent component (Table 1, Figure 1b) resulted in two clearly different components. While the low-affinity and high-capacity component was unaffected by tumour growth, the high-affinity, low-capacity component of the tumour-bearing rats showed an important increase in V_{max} (78%). However, for leucine transport, tumour burden induced important increases in the Na^+ -independent component, not only in K_m (262%) but

Table 1 Alanine transport in rat placental membrane vesiclesResults are mean values $\pm$ S.E.M. Na⁺-independent experiments used 4 independent vesicle preparations and Na⁺-dependent experiment used 3 preparations of each cell type.

	Na ⁺ -independent		Diffusion		Na ⁺ -dependent		
	K_m (mM)	V_{max} (pmol of alanine/mg per 5 s)			K_m — 1 (mM)	V_{max} — 1 (pmol of alanine/ mg per 5 s)	V_{max} — 2 (pmol of alanine/ mg per 5 s)
Control	2.01 $\pm$ 0.99	359 $\pm$ 100	153 $\pm$ 5		5.42 $\pm$ 0.83	4208 $\pm$ 223	387 $\pm$ 68
Tumour	2.42 $\pm$ 0.92	1050 $\pm$ 113**	70 $\pm$ 24		6.18 $\pm$ 0.79	3535 $\pm$ 153	690 $\pm$ 50*
TNF	1.40 $\pm$ 0.76	734 $\pm$ 156	104 $\pm$ 24		4.94 $\pm$ 0.35	4001 $\pm$ 87	648 $\pm$ 35*

Statistical significance of the results (Student's *t* test): * $P < 0.05$; ** $P < 0.01$; tumour or TNF versus Control.

also in V_{max} (189 %) (Table 2, Figure 2a). Interestingly, no changes were observed in relation to the Na⁺-dependent component of leucine transport (Table 2, Figure 2b).

TNF and amino acid transport

TNF, either administered directly or induced by lipopolysaccharide injection, has been demonstrated to cause placental haemorrhage and abortion in experimental animals [19]. This is partially explained by the fact that TNF has been shown to be cytotoxic for endothelial cells from human umbilical vein [20] and may damage the decidual vasculature. Additionally, the placenta contains TNF receptors localized in the trophoblast and TNF inhibits growth of the trophoblast, as measured by DNA metabolism and cellular proliferation [21].

Since elevated TNF concentrations have been reported in this kind of tumour model [22], and since the cytokine has been reported to have important metabolic effects [23], we performed the same type of transport experiments in rats chronically treated with TNF. The results obtained showed great similarities with those observed with tumour growth. The V_{max} of Na⁺-independent alanine transport was also increased by the cytokine (104 %) while no changes were observed in affinity (Table 1, Figure 1a). TNF treatment also induced an increase in the V_{max} (70 %) of the Na⁺-dependent component while no changes in affinity were observed (Table 1, Figure 1b). With regard to leucine kinetics, TNF treatment, as in the case of tumour growth, also increased K_m (155 %) and V_{max} (72 %) associated with Na⁺-independent transport (Table 2, Figure 2a). Interestingly, treatment with the cytokine increased both the K_m (43 %) and V_{max} (64 %) of the Na⁺-dependent component (Table 2, Figure 2b).

Inhibition of L-alanine uptake by a range of amino acids

In order to determine the properties of the systems responsible for the Na⁺-dependent uptake of alanine, the inhibition of Na⁺-dependent alanine transport (0.1 mM) was examined (Table 3). Similar inhibition patterns were observed between control and tumour membrane vesicles preparations. This initial finding suggests that the expression of the different transport systems involved in the L-alanine uptake by the microvillus was not altered by the presence of the tumour.

The presence of 10 mM alanine, cysteine or serine in the incubation medium produced a marked effect on L-alanine uptake, showing a percentage of inhibition greater than 90 %. In the case of methylaminoisobutyrate (MeAIB) and glutamine, the inhibitory effect was also quite important (75–80 %) and greater than that produced by leucine, 2-amino-2-norbornane carboxylic acid and serine (less than 35 %). The inhibitory pattern produced by MeAIB (specific substrate of the A system) suggests that this transport system is most likely the main system involved in the L-alanine uptake in these placental membrane vesicles. Also, the fact that the inhibitory effect of alanine itself is greater than that produced by MeAIB agrees with the presence of more than one Na⁺-dependent transport system for L-alanine. In fact, cysteine and serine inhibitory patterns (both substrates of the A and ASC systems) suggest the presence of the ASC system which may account for the 15–20 % of L-alanine uptake under these conditions.

Surprisingly, the presence of 10 mM leucine in the sodium medium produced a partial inhibitory effect (near 33 %) on L-alanine uptake. This could be explained by the likely presence of the B^o system, widely described in the renal epithelium and in the intestinal apical membrane [24] where it accounts for the majority of L-alanine uptake. Also, the data presented in Table 2 and

Figure 1 Kinetics of alanine transport in reconstituted placental membrane vesicles

Results are means $\pm$ S.E.M. of four different membrane vesicle preparations. Membrane vesicles were prepared as previously described in the Experimental section and were incubated in a medium containing different amino acid concentrations ranging from 0.1 to 20 mM in final concentration. (a) Na^+ -independent uptake. (b) Na^+ -dependent uptake.

Table 2 Leucine transport in rat placental membrane vesicles

Results are mean values $\pm$ S.E.M. with the number of independent vesicle preparations indicated in parentheses.

Na ⁺ -independent transport				Na ⁺ -dependent transport	
	<i>K_m</i> (mM)	<i>V_{max}</i> (pmol of leucine/mg per 5 s)	Diffusion	<i>K_m</i> (mM)	<i>V_{max}</i> (pmol of leucine/mg per 5 s)
Control	0.096 $\pm$ 0.014 (4)	104 $\pm$ 3 (4)	177 $\pm$ 4 (4)	0.171 $\pm$ 0.057 (3)	105 $\pm$ 11 (3)
Tumour	0.348 $\pm$ 0.047** (4)	301 $\pm$ 11*** (4)	124 $\pm$ 6 (4)	0.070 $\pm$ 0.035 (4)	96 $\pm$ 8 (4)
TNF	0.245 $\pm$ 0.071 (3)	179 $\pm$ 17***††† (3)	64 $\pm$ 7 (3)	0.244 $\pm$ 0.032† (3)	172 $\pm$ 8***††† (3)

Statistical significance of the results (Student's *t* test): ** *P* < 0.01; *** *P* < 0.001; Tumour or TNF versus Control; † *P* < 0.05; ††† *P* < 0.001; TNF versus Tumour.

Figure 2(b) seem to reinforce this idea since a Na^+ -dependent L-leucine transport is described. From all the data presented here we propose that, under these experimental conditions, a B^0 -like system is present and that it could account for 5–10% of the total L-alanine uptake.

Concluding remarks

Our results illustrate how the mother is able to adapt her metabolism to cope with the increasing nitrogen demand of the

exponentially growing fetus in the presence of a rapidly developing tumour. The results obtained are in agreement with those obtained previously in which the implantation of the Yoshida AH-130 hepatoma to pregnant rats resulted in no changes in fetal mass.

From the results of inhibition experiments, we can suggest that there are at least three Na^+ -dependent L-alanine transport systems (namely A, ASC and probably B^0) present in the microvillus placenta membrane, although further experiments should be done to confirm their presence.

Figure 2 Kinetics of leucine transport in reconstituted placental membrane vesicles

Results are means $\pm$ S.E.M. of four different membrane vesicle preparations. Membrane vesicles were prepared as previously described in the Experimental section and were incubated in a medium containing different amino acid concentrations ranging from 0.1 to 10 mM in final concentration. (a) Na^+ -independent uptake. (b) Na^+ -dependent uptake.

Table 3 Inhibition of Na^+ -dependent alanine uptake by a range of amino acids

Inhibitor (10 mM)	Percentage of inhibition	
	Control (4)	Tumour (3)
Leu	32 $\pm$ 4	34 $\pm$ 3
BCH	11 $\pm$ 3	10 $\pm$ 3
Gln	76 $\pm$ 3	80 $\pm$ 1
Ser	92 $\pm$ 1	93 $\pm$ 1
Cys	94 $\pm$ 2	93 $\pm$ 1
Lys	16 $\pm$ 6	27 $\pm$ 6
MeAIB	77 $\pm$ 2	76 $\pm$ 1
Ala	92 $\pm$ 1	94 $\pm$ 1

The results are expressed as percentage of inhibition $\pm$ S.E.M. for the number of membrane vesicle preparations indicated in parentheses. Transport of alanine (0.1 mM) was measured over 5 s in the presence and absence of Na^+ as described in the Experimental section. The inhibition of Na^+ -dependent uptake was determined as follows: $I = 100 - [(V_i^{\text{Na}} - V_i^{\text{K}})/(V_i^{\text{Na}} - V_i^{\text{K}})] \cdot 100$ where I represents the percentage of inhibition caused, V_i^{Na} and V_i^{K} represent the uptake rate in the presence or absence of sodium respectively, and V_i^{Na} and V_i^{K} represent the uptake rate in the presence of the inhibitor amino acid. BCH: 2-amino-2-norbornane carboxylic acid.

In addition, our present work also confirms that TNF can mimic many metabolic responses associated with tumour growth, as has previously been reviewed [23]. Chronic TNF administration to pregnant rats results in very similar changes in alanine

and leucine transport kinetics, these results suggest that the cytokine may mediate the host response in response to tumour growth.

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