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Remission of hepatocellular carcinoma with arginine depletion induced by systemic release of endogenous hepatic arginase due to transhepatic arterial embolisation, augmented by high-dose insulin: arginase as a potential drug candidate for hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is auxotrophic for the semi-essential amino acid arginine, depletion of which leads to tumor death. In humans, arginine is not an essential amino acid since many adult somatic cells can re-synthesize it from other sources, such as citrulline. Enzymes capable of depleting arginine in vitro include the urea cycle enzyme arginase, which is found in abundance in human liver. For over three decades, arginase has not been considered as a potential drug candidate because of its low substrate affinity, short circulatory half-life and sub-optimal enzymatic activity at physiological pH, though its in vitro anti-tumor activities in certain tumors have been amply reported. Arginine deiminase, a bacterial enzyme from *Mycoplasma hominus* has been shown to induce HCC remission through the mechanism of arginine depletion.

We report here an innovative treatment approach for the treatment of locally advanced and metastatic HCC with transhepatic arterial embolisation (TAE) of the liver tumor with lipiodol and gel foam as a means of inducing a leakage of hepatic arginase from the liver into the circulation. Hepatic arginase released into the systemic circulation rapidly depleted plasma arginine. High-dose insulin was included to induce a state of hypoaminoacidaemia to augment arginine depletion. With this protocol, we have treated seven patients with locally advanced and/or metastatic HCC. Five patients achieved arginine depletion, ranging from 0 to 20 μM (normal plasma level 100–120 μM); all had varying degrees of tumor remission in their primary tumors and extra-hepatic sites in the lymph nodes, lungs and bones, suggesting systemic anti-cancer effect of arginine depletion. The two non-responders did not show significant reduction in plasma arginine. Based on our findings, we propose that the urea cycle enzyme, arginase, is a good drug candidate for the treatment of HCC.

Keywords: Arginase; Arginine depletion; Auxotrophic; Hepatocellular carcinoma; Transhepatic arterial embolisation; High-dose insulin infusion

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1. Introduction

For cells to grow, whether normal or malignant, nutrients have to be in constant supply. The high arginine requirement of malignant cells for growth has been reported for over a century [1]. Restriction of amino acid, in particular arginine, seems a rational way of controlling cell growth since arginine is featured in a plethora of metabolic pathways [2]. Not only does it serve as a building block for peptides and protein synthesis, it is also a precursor for creatine, polyamines, glutamine, glutamate, proline and a number of neurotransmitters including GABA. It is also a substrate for arginase, which is the key enzyme in the urea cycle in the detoxification of ammonia and nitric oxide synthases (NOS) in the generation of nitric oxide, which has been a subject of intense scientific research in the last two decades [3]. It is an essential amino acid in developing embryo, growing vertebrates and certain tumor cells. Arginine requirement is elevated in acute stress such as sepsis and wound healing [1]. Arginine, however, is not an essential amino acid in adult humans. In fact, arginine free diet is compatible with healthy growth since adult somatic cells can re-synthesize arginine from other sources, such as citrulline from the intestinal–renal axis and glutamine, glutamate and proline from the small bowel [4]. Citrulline in the circulation is converted to arginine in the somatic cells and notably in the proximal tubules of the kidney via a two-step enzymatic process involving the enzymes argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL) [5].

For certain tumors, in particular liver cancer, melanoma and sarcoma, arginine is an obligatory essential amino acid, restriction or depletion of which will lead to cell death [6]. These tumors are said to be 'auxotrophic' for arginine since they cannot internally re-synthesize arginine from citrulline.

During the 1970s and 1980s, the urea cycle enzyme arginase seemed a promising drug candidate since it exhibited marked in vitro cytotoxic activity against tumor cells. Storr and Burton [7] were the first to demonstrate the relationship of the level of arginine depletion in culture and cancer death. The authors showed that mouse lymphoma cells in Fisher's medium (80 μM arginine) treated with liver extract, which is a rich source of arginase, died within 6–24 h. Normal lymphocytes and fibroblasts treated in the same manner would cease to go into cell division. The level of arginine depletion was dependent on the amount of arginase put into the medium and it appeared that when arginine level in the medium dropped <8 μM for over 24 h, irreparable cell death would occur in lymphoma cells even when arginine was replenished into the medium. On replenishing arginine, normal lymphocytes and fibroblasts would resume normal cell division, apparently unharmed. The authors also showed that such anti-tumor activity of arginase was fully reversed with arginine supplement and partially with citrulline and argininosuccinic acid, i.e. other components of the urea cycle. The authors attempted to deplete arginine in vivo with purified arginase from bovine liver extract given intraperitoneally to mice bearing L1210 and L5178Y ascitic tumors. Although the ornithine level in ascitic fluid was found to be elevated, signifying arginase activity, no demonstrable anti-tumor effect was observed. Since ornithine can be converted back to arginine in situ in the peritoneal cavity, it is not surprising that no anti-tumor on L1210 and L5178Y was seen. This is believed to be the first in vivo attempt at arginine depletion with arginase, and sowed a seed of doubt that arginase held any promise as an anti-cancer agent.

Savoca et al. [8] reported the second attempt at in vivo arginine depletion with native bovine arginase. This time, realizing its short in vivo half-life, the authors conjugated the bovine arginase with a monomethoxypolyethylene glycol (PEG), a process called 'pegylation'. Pegylation was first introduced in the 1980s to increase the molecular size of a protein/enzyme by attracting water molecules to its lysine residues so as to prolong its half-life by decreasing its clearance in the blood, hopefully without affecting too greatly its enzymatic activity [9,10]. When injected into experimental mice bearing Taper liver tumor, the authors observed no obvious anti-tumor activity. They then concluded that PEG-arginase had no in vivo anti-cancer activity, although the animals survived longer than the control group. They were unable to measure the plasma levels of arginine in these experimental animals to detect the extent to which arginine depletion had occurred. In terms of biochemical characteristics, arginase was not considered a good drug candidate for at least three main reasons, namely,

its low substrate affinity, short plasma half-life, and low enzymatic activity at physiological pH [7,8,11–15]. These views are still widely held in the scientific community nowadays [16].

In early 2001, the first author witnessed a case of possible iatrogenic arginine depletion. The case was a 50 year-old male patient with locally advanced HCC. In early February 2001, the patient had an episode of acute and massive hepatic rupture leading to haemoperitoneum. Out of curiosity, the senior author (PNMC) tested the patient's plasma for arginine levels, which were found to be almost undetectable (0–3 μM) for >3 days and his peritoneal blood arginine was also low at ~ 7 μM during this period. It was postulated that the hepatic arginase released from his ruptured liver was the reason for the systemic arginine depletion. This period of arginine depletion was followed by normalization of his liver cancer marker alpha foetal protein (AFP), which dropped from 86 to 8 ng/ml in just over 2 weeks. The patient also had a period of improvement in this clinical performance. His AFP remained normal at <10 μM until he died 4 weeks later from liver failure, probably secondary to liver cirrhosis unrelated to his liver cancer.

The normalization of AFP and improvement in clinical well being of this index case gave the author the conviction that systemic arginine depletion had occurred due to the endogenous release of hepatic arginase from the ruptured liver and that such arginine depletion could have led to biochemical remission of his liver cancer. It then came to our understanding, after communicating with Professor Ikemoto (University of Kyoto, Japan) that 'endogenous' release of hepatic arginase occurs in a number of pathological conditions [17–20]. These include liver injuries induced by hepatectomy, cryoablation, post-orthotropic liver transplantation, graft rejection after liver transplantation, and in fact any form of arterial perfusion defect in the liver such as clamping of the hepatic arterial supply before orthotropic liver transplantation. We also speculated that transhepatic arterial embolisation (TAE), which is also a form of arterial perfusion defect, may also cause a leakage of hepatic arginase from the hepatocytes into the circulation. This, however, had never been shown or reported.

A clinical experiment was designed to test the hypothesis that endogenous hepatic arginase was released into the systemic circulation with TAE, which is a recognized form of treatment in patients with unresectable or advanced liver cancer [21,22]. However, the amount of endogenous arginase released from TAE was likely to be low and unsustained to have any major impact on the plasma arginine level. The notion that high-dose insulin infusion might be of use in inducing a state of 'hypoaminoacidaemia', as reported by a group at the Memorial Sloan-Kettering Cancer Center in New York [23], and that such hypoaminoacidaemia may augment the effect of arginine depletion by shifting down the baselines of all the branch-chain amino acids, of which arginine is one. The hypoaminoacidaemic effect of insulin is thought to be due to the promotion of uptake of amino acids into the cells and inhibition of muscle breakdown [23]. These were the rationales for using high-dose insulin by the Sloan Kettering group to try and alleviate cachexia in cancer patients.

In the present study, we designed a clinical experiment to test the following hypotheses:

(1) Endogenous arginase could be induced to release into the systemic circulation with TAE.
(2) Plasma arginine levels could be lowered by simultaneous infusion of high dose insulin so as to augment the arginine depleting effect of endogenous arginase so released from TAE.
(3) If arginine depletion was achieved, it would induce systemic remission of liver cancer.

2. Materials and methods

2.1. Patient selection and preparation

In total, seven patients with unresectable and locally advanced liver cancer were recruited into the study and treated with TAE and high dose insulin infusion. All patients gave written consents to TAE and infusion of insulin/dextrose in accordance with the declarations of Helsinki.

Inclusion criteria were as follows:

(1) Histologically confirmed HCC
(2) >18 years of age
(3) KPS $\geq 60\%$
(4) Life expectancy of >12 weeks

(5) Of conscious mind to sign informed consent as per Helsinki Declaration
(6) No systemic chemotherapy for at least 4 weeks
(7) CT/PET measurable disease
(8) Elevated pretreatment alpha fetal protein (AFP) levels
(9) No evidence of portal vein thrombosis
(10) Total bilirubin <2.0 mg/dl
(11) Serum GOT $<5\times$ upper limits
(12) Platelet count of $>100{,}000$.
(13) Non-diabetic

Pretreatment work-up included a full physical examination, chest X-ray, CT scan of the abdomen and other involved extra-hepatic sites, complete blood picture, full liver and renal function tests, prothrombin time and AFP. On the day of admission, a central venous catheter was inserted via a superficial neck vein for venous access. The day before transhepatic embolisation, they were started on the high-dose insulin (90 units of actrapid insulin per day). Dextrose (50% w/v) via central venous catheter infusion was given to maintain normo-glucaemia with a sliding scale insulin.

2.2. Transhepatic arterial embolisation

On the day of hepatic angiography, the patients had already been on high-dose insulin/dextrose 50% and sliding scale insulin for at least 12 h. Standard hepatic angiography was performed under strict asceptic setting, using the right groin approach. Once the hepatic artery was cannulated, contrast dye was injected to locate the hepatic tumor 'flash'. About 50 ml of Lipiodol and gel-foam in sufficient quantities were injected intra-arterially until no arterial perfusion of the feeding artery was seen. Blood samples were taken before, during and after completion of TAE for arginase assays and arginine levels.

2.3. Blood sampling and ELISA assay

Throughout the treatment period, blood samples were taken on a daily basis to assess the arginase and arginine levels. The morning blood samples were tested for CBC, LFT, RFT, full clotting studies and AFP levels. Blood samples were collected in pre-cooled tubes and serum samples separated and stored at -20 °C before sending them to Dr Ikemoto (Kyoto University) for arginase assessment using his ELISA assay [17].

2.4. Measurement of arginine levels in blood plasma

For measuring arginine levels, amino acid analysis (AAA) was performed using a high-speed amino acid analyzer (Hitachi, L-8800) with an ion-exchange column, reactor temperature at 135 °C and photometer at 570 nm for detection. The column, standards, buffers, and all other reagents were purchased from and used strictly according to the manufacturer's (Hitachi) recommended protocols. Briefly, 1 ml whole blood samples were taken from the patients daily. Blood samples were centrifuged at 14,000 rpm for 5 min. After this, 400 μl of plasma was well mixed with 400 μl 50% (w/v) trichloroacetic acid (TCA) and incubated on ice for 10 min. The precipitated mixture was centrifuged at 14,000 rpm for 10 min and the clear supernatant was analyzed with an amino acid analyzer according to the manufacturer's instruction.

2.5. Measurement of arginase activity in blood plasma

One milliliter whole blood sample of the TAE treated patient was taken daily. Blood samples were centrifuged at 14,000 rpm for 5 min to collect the blood plasma. The plasma (200 μl) was incubated at 37 °C for 30 min before 100 mM (final concentration) arginine was added to the plasma, and the mixture further incubated at 37 °C for 30 min. Finally, 200 μl of 50% (w/v) TCA was added and the sample was centrifuged at 14,000 rpm for 10 min. The clear supernatant was analyzed by the amino acid analyzer for the detection of arginine and the product ornithine.

The activity of arginase is calculated using the following equation:

$$\text{Activity} = \frac{\ln\left\{\frac{C_{\text{arg}(i)}+[C_{\text{arg}(i)}+C_{\text{orn}(i)}]-[C_{\text{arg(i)}}+C_{\text{orn(i)}}]}{C_{\text{arg(f)}}}\right\}}{\text{Reaction time}}$$

where $C_{\text{arg(i)}}$, initial concentration of arginine, $C_{\text{arg(f)}}$, final concentration of arginine, $C_{\text{orn(i)}}$, initial concentration of ornithine, $C_{\text{orn(f)}}$, final concentration of ornithine.

2.6. Post-treatment evaluation

Positron emission tomography (PET) and CT scans of abdomen and any extra-hepatic sites were taken 4 weeks after treatment to assess structural and functional changes to the primary tumors in the liver and metastatic sites. AFP levels were taken initially daily and then weekly to monitor the treatment progress.

2.7. Definitions

- Adequate Arginine Depletion: Arginine level $\leq$10 μM over 24 h
- moderate arginine depletion: 10 μM $<$Arginine level $\leq$40 μM over 24 h
- no arginine depletion: arginine level $>$40 μM with or without any detectable arginase level
- complete remission (CR): complete disappearance of all clinical, radiological detectable disease, no FDG uptake on PET scanning with normalization of the disease marker AFP
- major response (MR): $>$50% reduction in bidimensionally measurable disease on CT or $>$50% reduction in FDG uptake on PET
- partial response (PR): $<$50% reduction in bidimensionally measurable disease or less than 50% reduction in FDG uptake on PET
- progression of disease (POD): clinical, radiological or biochemical sign of an increase in disease activity.

3. Results

In total, seven patients with locally advanced and metastatic HCC were recruited, six males and one female with ages ranging from 44 to 65 years. All had measurable disease in their liver on CT and elevated AFPs pre-treatment. Two had extensive disease in the liver only with no extra-hepatic lesion (cases 2 and 7). Five had local disease in the liver as well as extra-hepatic disease: one with portal lymph nodes (case 1), 1 with extensive mesocolic metastases, para-aortic adenopathy and multiple disseminated bone metastases (case 3), two with extensive retroperitoneal lymph nodes only (cases 4 and 5) and one had bilateral pulmonary nodules (case 6).

In all the cases studied, plasma arginine baselines fell to about 50–60 μM with high dose insulin infusion. With TAE, there were upsurges of plasma arginase, as detected by both arginase activity and ELISA assays (activity and protein assays), which would last for 2–3 days. There were corresponding troughs of arginine in the plasma, mirror-imaging the arginase peaks.

Five patients had documented arginine depletion, one defined as moderate (Fig. 1A) at 30–40 μM (case 1) and four adequate with arginine levels between 0 and 10 μM (cases 2, 3, 6 and 7). One (case 3) had undetectable arginine level for over 2 days (Fig. 1B). Arginine depletion was a pre-requisite for systemic tumour response, i.e. tumour shrinkage outside the embolisation field (Table 1).

In case 1 and case 3, blood arginine levels dropped from100 μM to just above 80 μM with high dose insulin. On TAE, there was a precipitous drop in the arginine level, corresponding to upsurge of arginase level. Arginine depletion in case 1 was moderate, nadir at 30 μM level whereas in case 3, no detectable arginine was seen lasting for 48 h. In the amino acid analysis plots, pre-TAE arginine and ornithine peaks were clearly seen. With embolisation, arginine peak disappeared and there was a corresponding increase in the ornithine spike suggesting enzymatic conversion of arginine to ornithine (Fig. 2). In both cases, tumor remissions were seen 4 weeks after TAE. Though arginine depletion was not as profound in case 1 as in case 3, complete response was seen in case 1 and only a mixed response was seen in case 3. This suggests that different HCC may have different sensitivity to arginine depletion.

One patient (case 1) achieved complete remission with disappearance of all radiologically detectable disease on CT and PET on week 4. Radiologically, his right lobe disease became completely necrotic on week 4. More importantly, the original left lobe satellite tumor nodules, which were quite evident on pre-treatment CT and PET scans, also disappeared on the week 4 scan. Complete tumor remission was also evident on PET with no uptake of FDG in the primary site in the right lobe of the liver, left lobe lesions and portal lymph nodes on week 4 (Figs. 3 and 4). On subsequent follow up scan in October, four months

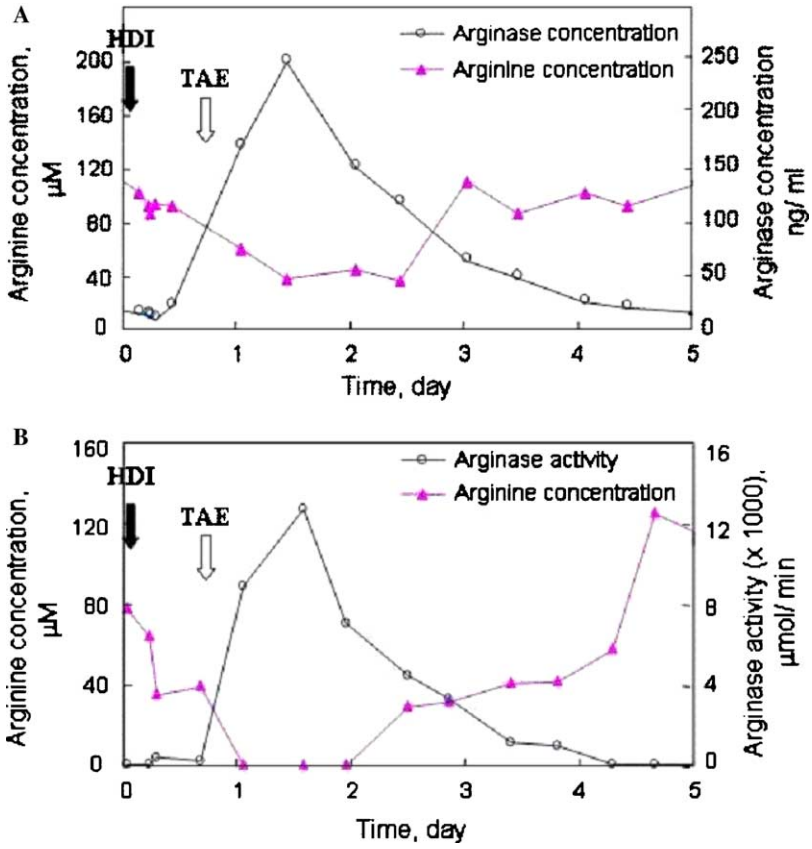

Fig. 1. (A) Response of patient (case 1) treated with TAE. High dose insulin (HDI) infusion, 90 units per 24 h of Actrapid, was started at time 0. Note the arginine level in blood dropped from just below 120 μM to just above 80 μM. On embolisation, endogenous arginase was released into the systemic circulation (as measured by ELISA tests). Plasma arginine level continued to drop to around 40 μM and stayed the same for about 24 h before moving up again, corresponding to the decrease in amount of arginase released. (B) Response of patient (case 3) treated with TAE. On embolisation, endogenous arginase was released into the systemic circulation, as measured by arginase activity assay. Plasma arginine level continued to drop to undetectable level and stayed the same for about 24 h before moving up again, corresponding to the decrease in amount of arginase released.

after treatment, the large right lobe tumor had undergone cystic changes with no evidence of recurrence in the left lobe and lymph nodes in the portal hepatitis. There was also normalization of cancer marker AFP (Fig. 5), which lasted for over 5 months, after which his AFP began to rise. He was lost to follow-up at 12 months.

The other four patients had partial remissions of their primary tumours in the liver lasting between 1 to 3 months. Two patients (cases 3 and 6) had remission of disease in lungs, omentum, retroperitoneal lymph nodes, all outside the embolisation fields. The other two patients (cases 2 and 7) had no extrahepatic disease. Case 2 had major response in his liver on PET criteria and improvement in clinical well being, but died of disease progression in 6 months. Case 7 who had a major response in his liver underwent radio-frequency ablation of his residual liver tumor, and is still alive and well in complete remission of his HCC at time of writing this manuscript, disease-free 3 years after his initial TAE.

All patients had normal blood pressure readings throughout treatment and their laboratory tests, including platelet counts and clotting functions, remained within the normal limits throughout the study (results not shown). The clinical outcomes, AFP changes, radiological and PET responses and survival status of these seven patients are tabulated in Table 1.

Table 1
Summary of the seven cases treated with TAE and high-dose insulin

		AFP response	Radiological response	PET response	Remarks	Survival outcome
Case 1 M/65	Moderate adequate	Normalization lasted for 8 months	CR in primary and portal lymph nodes	CR on PET in primary tumor and portal nodes	Normal KPS for 8 months	Still alive and well in CR at time of lost to follow up in 8 months
Case 2 M/50	Adequate (<10 μM)	Over 70% drop in AFP in 2 weeks	Minimal response in primary tumor, no extra-hepatic disease to evaluate	MR on PET in primary tumor site	Improved KPS	Died of POD in 6 months
Case 3 M/44	Adequate (zero)	Normal AFP for 8 weeks	MR in liver and extra-hepatic disease	MR in liver and extra-hepatic sites in para-aortic and mesenteric nodes	Improved KPS	Died of aspiration pneumonia, in complete remission in 3 months
Case 4 F/44	No depletion	Over 75% drop in AFP in 4 weeks	Minimal response in primary tumor	PR in liver, POD in extra-hepatic sites, therefore mixed response	Improved KPS, had second TAE/HDI with no response	Died of POD in 6 months
Case 5 M/48	No depletion	Over 50% reduction of AFP	PR in liver, POD in extra-hepatic sites	NO response on PET	Stable KPS	Died of POD
Case 6 M/56	Adequate (<10 μM)	Over 75% reduction	PR in primary tumor site	Minor decrease in lung activity	Improved KPS	Residual disease ablated with RFA, well in CR, lost to follow up at 8 months
Case 7 M/68	Adequate (<10 μM)	Over 90% reduction	MR in primary tumor site	MR in liver on PET	Improved KPS	Residual disease resected, in CR for 3 years

4. Discussion

In the present study, we have confirmed our first hypothesis with ELISA assays and arginase activity tests that systemic release of hepatic arginase was possible with transhepatic arterial embolisation (TAE). With the shifting of the baseline arginine levels with high dose insulin infusion, arginine depletion with endogenous hepatic arginase was possible and in fact greatly facilitated, as in case 3, thus confirming our second hypothesis. The associated enzymatic conversion of arginine to ornithine was seen in the plasma samples of the five patients who achieved arginine depletion as evident by the greatly increased ornithine peaks and absence of arginine peaks during treatment (Fig. 2). This supports our third hypothesis that enzymatic conversion of arginine to ornithine took place in the circulation with the endogenous hepatic arginase so released from the liver, which is the only organ in the body rich in the enzyme. From the pattern of response, one can conclude that arginine depletion, whether moderate or complete, is a pre-requisite for systemic tumour remission and that such arginine depletion was a direct consequence of endogenous hepatic arginase so released.

The role of high dose insulin infusion appeared to be critical in augmenting the arginine depletion process, at least in our study. The actions of insulin were to enhance the uptake of all the branched chain amino acids into the cells and inhibit muscle catabolism. In so doing, the baseline levels of all the amino acids, particularly all the branch-chain amino acids, including arginine, were lowered. These, in fact, were the findings reported by a Memorial Kettering group in New York when they tried to halt cancer cachexia by preventing muscle catabolism with high-dose insulin infusion [23]. It is tempting to

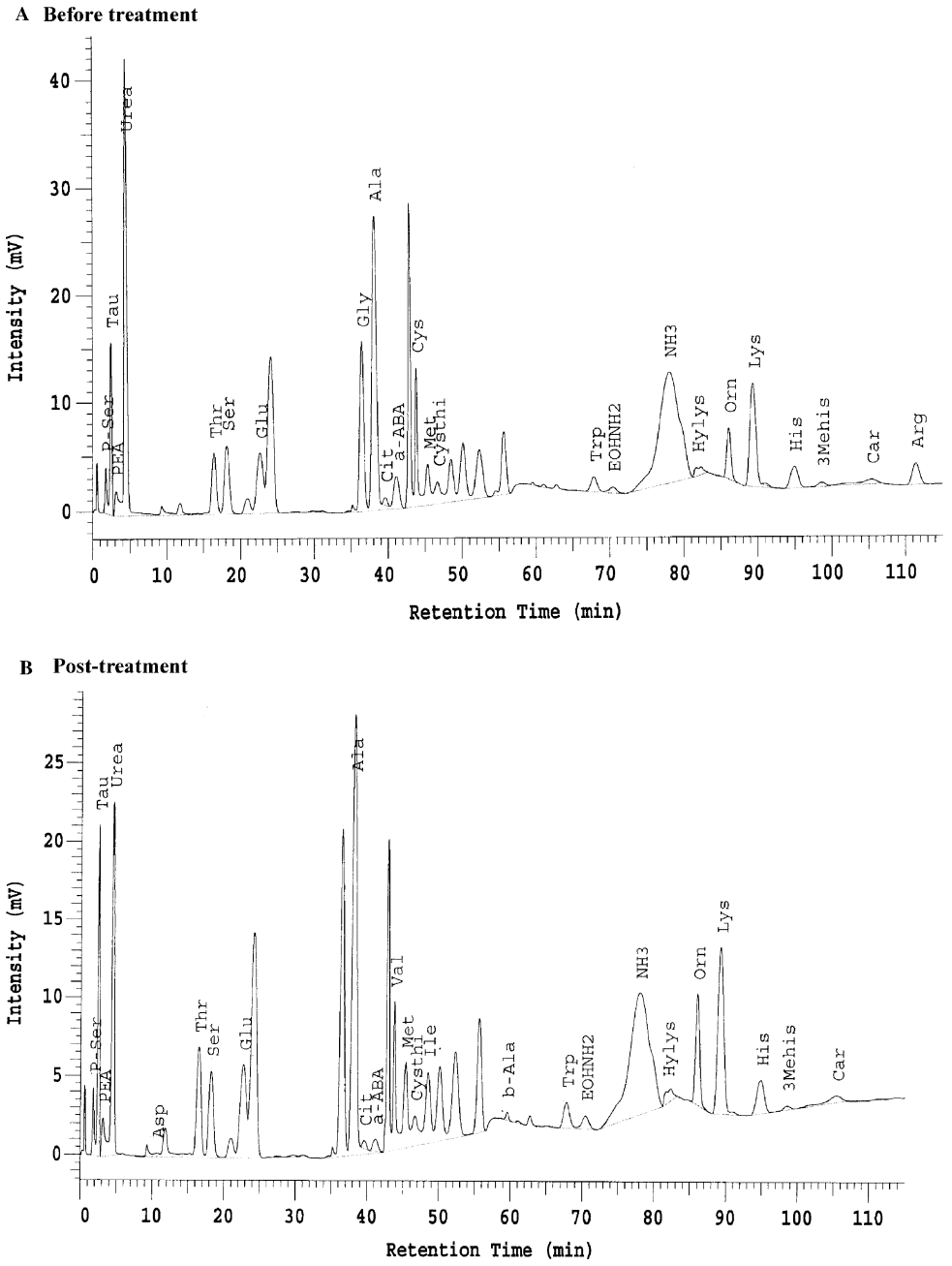

Fig. 2. Amino acid profiles of case 3. The arginine peak (Arg) disappeared after the TAE treatment. The ornithine peak (Orn) increased post-treatment.

speculate that this is the reason why, even with thousands of TAEs performed annually across the world for HCC, no report has indicated systemic tumour to TAE. The role of insulin in this type of treatment approach needs to be further clarified and confirmed.

We further showed in our study that the amount of endogenous arginase released paralleled with that of

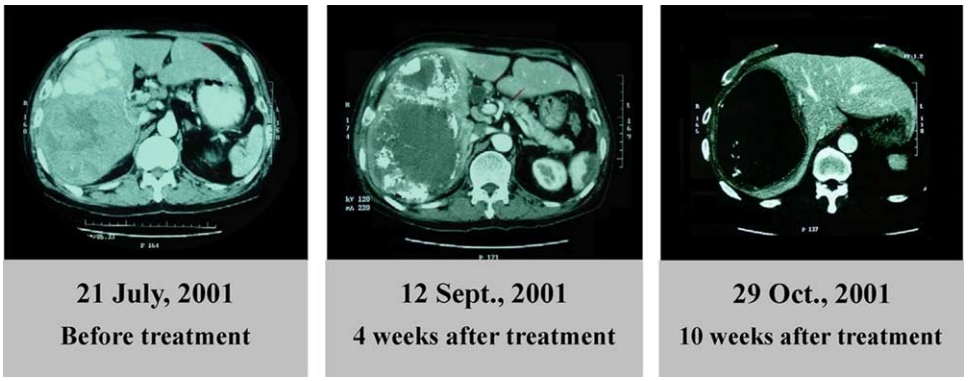

Fig. 3. CT scan for liver cancer patient (case 1). Serial computerized tomograghic images of the liver tumor (mainly in right lobe and scattered smaller nodules in the liver lobe) before treatment, 4 weeks after treatment and 10 weeks after treatment showing cystic transformation of the liver tumor with no evidence of residual disease in right and left lobe. The October scan (10 weeks after treatment) showed regeneration of left lobe and large cyst in right lobe with widest diameter over 18 cm.

the hepatic transaminase sGOT. In fact, the sGOT levels correlated so well with the amount of endogenous arginase released that it could be used as a surrogate for the arginase release (results not shown). This perhaps is not surprising since sGOT and arginase are mitochondrial in origin, and may leak out of the traumatized hepatocytes at the same time. It is important to note also that all the patients had normal blood pressure readings and haematological parameters such as platelet and clotting studies throughout the treatment period (results not shown). Although we did not study the patients' blood nitrate and nitrite levels, which are of great relevance since nitric oxide is intimately related to platelet aggregation and endothelial vascular tone, the normal blood pressure readings and normal haematological parameters strongly suggested that nitric oxide production was not diminished with systemic arginine depletion. It can be theorized that since arginine could be re-synthesized from citrulline locally in the vascular endothelium, the nitric oxide at the endothelial microenvironment could still be maintained to exert adequate vascular tone. This, of course needs further elucidation when future arginine depletion studies are planned.

Systemic arginine depletion in the treatment of liver cancer has its obvious advantage since liver tumor derives over 90% of its blood supply from the hepatic artery, any systemic arginine depletion will deal a first blow to the liver tumor, which is auxotrophic for arginine [32]. At least in the first case studied, arginine depletion led to durable tumor remission of over 5 months as shown by the normalization of the AFP readings, total clinical well being and remission of extra-hepatic disease, which cannot be accounted for with TAE alone. It is interesting to note also that PET is more sensitive than CT in detecting smaller degrees of tumor response. This can be explained by the fact that PET can detect small degrees of metabolic changes well before obvious shrinkage of size of the tumor on CT, which is only an imaging scan.

Our present finding of systemic liver cancer remission with endogenous arginase begs the obvious question of whether arginase itself, say, in recombinant form given systemically, could be a potential drug candidate for the treatment of HCC through its mechanism of arginine depletion.

There are at least two ways in which arginine can be depleted from circulation in vivo, physical and enzymatic. A group in Zurich used extra-corporeal dialysis with an arginine depleting enzyme, such as bovine arginase or autologous liver extract, added in the secondary circuit to deplete the arginine in the blood returned to the experimental dogs [24]. This method is cumbersome and impractical for human use, not to mention the metabolic upsets dialysis could induce in a sick cancer patient. As for enzymatic depletion, there are at least three enzymes that are capable of depleting arginine both in vitro

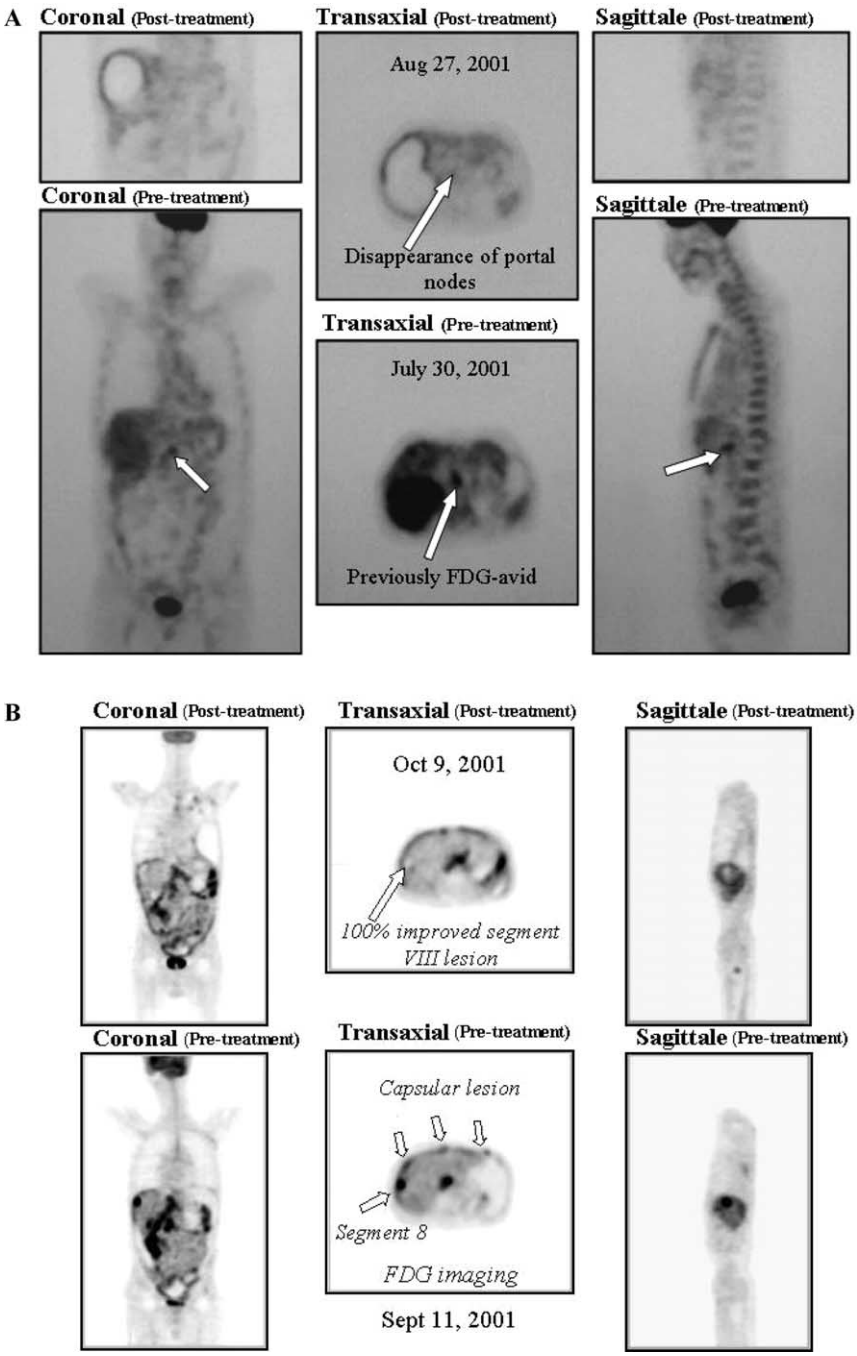

Fig. 4. (A) PET scan of case 1. Just before embolisation (pre-treatment) and 4 weeks after treatment (post-treatment). Note the 'total evacuation' of the liver tumor resulting in a 'cyst'-like structure. More importantly, the extra-hepatic metastatic lymph node in the portal region (therefore outside the embolisation field) also disappeared after treatment, signifying a systemic response to arginine depletion. (B) PET scan of case 3. Four weeks after embolisation of the liver (post-treatment). Note the extra-hepatic improvement of liver cancer on right mesocolic metastasis and paracaval nodes which were outside the embolisation field.

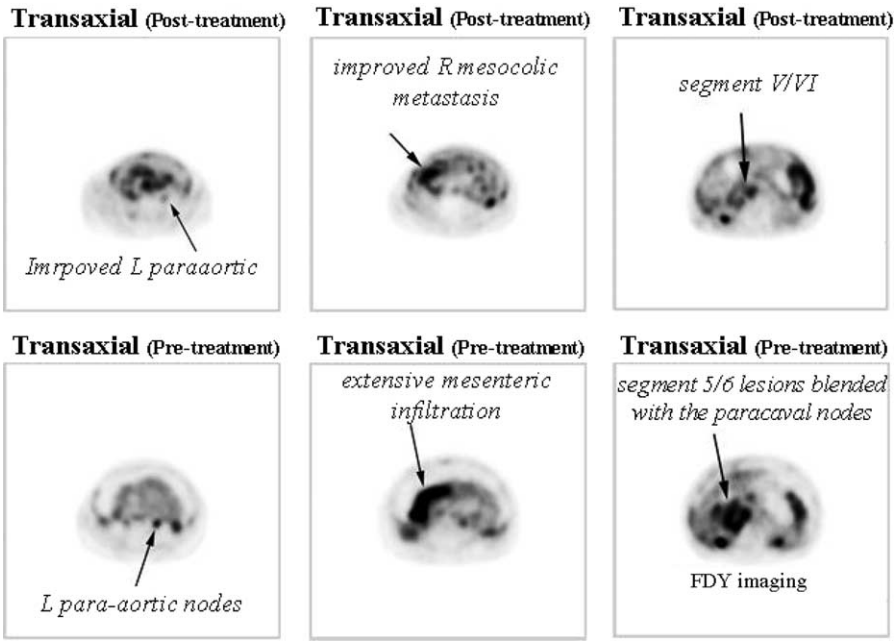

Fig. 4 (*continued*)

and in vivo, to varying efficacies. These are arginine decarboxylase, arginine deiminase (ADI) and the urea cycle enzyme arginase.

Arginine decarboxylase converts arginine to agmatine, which is toxic at high plasma concentration. It is also a bacterial derivative and will be immunogenic. Although Arginine decarboxylase has a favorable substrate affinity, it has a very short half-life, which is not amenable to pegylation since the arginine decarboxylase molecule has very few lysine residues to which polyethylene glycol can attach [1]. Furthermore, upon pegylation it looses virtually all its enzymatic activities. In practical terms, this renders arginine decarboxylase quite ineffective as an arginine-depleting enzyme.

Arginine deiminase has been shown to have strong in vitro and in vivo anti-HCC activities [25]. It is highly active against malignant melanoma too [26]. It is a bacterial enzyme derivative from *Mycobacterium arginini* [25,26] and it depletes arginine, mole for mole, to citrulline and ammonia. While citrulline can be converted back to arginine in somatic cells, ammonia is toxic and at high levels can cause hepatic pre-coma and encephalopathy, particularly when used in patients with compromised liver function (e.g. cirrhotics). The drug is a bacterial derivative and therefore immunogenic. Antibodies to ADI were detected as soon as week 2 after administration [27], even though pegylation can diminish this to a considerable extent. Despites these shortcomings, the drug ADI-PEG is currently in phase II and III clinical studies in Italy and MD Anderson Cancer Center in Texas, USA [27,28]. The recent publication of the Italian phase II data on the treatment efficacy of ADI-PEG on HCC is very exciting [27] with overall response of 47% (2/19 complete responses and 7/19 partial responses). Like many phase II studies, the initial optimistic response may not pan out in subsequent confirmatory randomized phase III studies. Therefore, until phase III studies are available one should interpret the present ADI-PEG results with cautious optimism.

The third enzymatic candidate is the urea cycle arginase. It has been considered in some reports [7,11–14,29,30] as ineffective in depleting plasma arginine in vivo for three main reasons. First, its short half-life of 10–15 min means vast quantities of the enzyme are required for effective depletion. Second, it

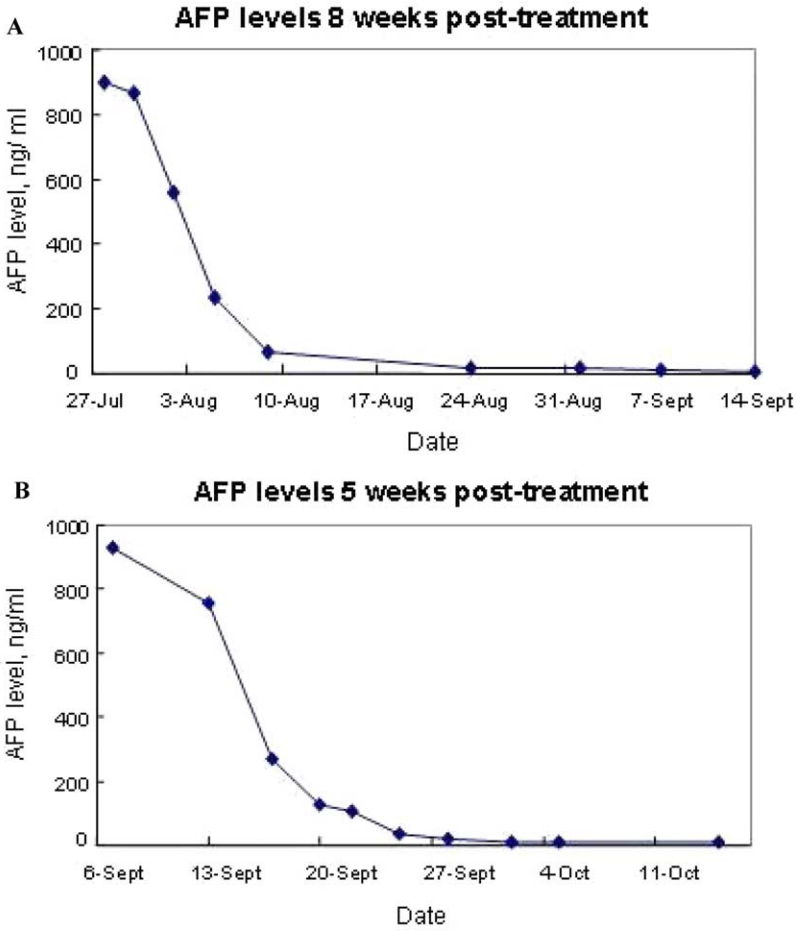

Fig. 5. (A) AFP response of case 1.The cancer marker AFP dropped from 860 to 8 ng/ml in ten days after the treatment. The normalization of AFP was lasted for 5 months post-treatment. (B) AFP response of case 3.

is a weak enzyme with low affinity for arginine (a high K_m of 2–4 mM); and third, its suboptimal enzymatic activity at physiological pH (optimal pH 9.3) would suggest a weak arginine-depleting activity in the blood circulation. Despite all these purported shortcomings, we have clearly demonstrated in our present study that arginase endogenously released from the liver does deplete plasma arginine efficiently at physiological pH and at such a 'low' substrate affinity. It is surprising that the above criticisms have been leveled at it because one would not expect the body would have evolved with such an enzyme in operation if it was so 'sub-optimal' in its performance. Since endogenous arginase induces effective arginine depletion, exogenous arginase (e.g. in a human recombinant form), should in theory be just as effective in inducing cancer remission.

Biochemically, the short half-life of recombinant human arginase can be lengthened by pegylation. Since there is no lysine residue at its enzymatic site, moderate pegylation does not affect to any significant extent its enzymatic activity. With pegylation, plasma clearance of the conjugated arginase is greatly reduced, increasing its circulatory half-life without affecting its enzymatic activity. Indeed, pegylation is now widely used in pharmaceutical industry to lengthen the circulatory half-lives of drugs, such as GCSF and interferon [9,10].

In our opinion, arginase has at least three other advantages over ADI-PEG. First, it is a human hepatic

enzyme and should therefore have low immunogenicity. Second, since arginase converts arginine to ornithine and urea, there is no ammonia toxicity as with ADI-PEG. And third, it takes even longer to recycle ornithine to arginine than citrulline. Hence, it may be superior to ADI-PEG.

At the Hong Kong Polytechnic University we have now developed a fully humanized recombinant hepatic arginase using recombinant DNA technology with *Bacillus subtilis* as the host for overexpression. Our research findings in the last two years have now demonstrated conclusively that pegylated recombinant human arginase is safe and effective in depleting plasma arginine level to zero in experimental rats for up to 3 months. Mostly importantly, in nude mice bearing human liver cancer xenografts, such as Hep 3B, Hep G2 and Huh 7, pegylated recombinant human arginase has significant tumor retarding activities in vivo (manuscript in preparation). Unlike ADI-PEG, pegylated recombinant human arginase may have a wider anti-cancer spectrum, which we have now been observed in our nude mice bearing other human tumor xenografts, including breast and colorectal and other cancers. On the other hand, ADI-PEG has no reported activities in these tumor types. This may well be due to the different drug mechanism that arginase has since it converts arginine to ornithine, which cannot across the cell membrane as efficiently as citrulline, making it more difficult for tumour cells to regenerate arginine from ornithine [31].

At present there is no standard treatment for locally advanced or metastatic liver cancer, a major health issue in China, South East Asia and Japan. In general, survival outcomes in these patients are bleak with average survival seldom more than a few months. Since chemotherapy, whether single agent or in combination, has not been shown to have any survival benefit in these patients and most cytotoxic agents have high toxicity profiles, any treatment modality with low treatment toxicity, such as arginine depletion, is most welcome. We expect pegylated recombinant human arginase to be at least as efficacious as ADI-PEG in inducing liver cancer remission and that it may have a wider anti-cancer spectrum, which needs to be confirmed in future preclinical studies. Its lack of ammonia toxicity and low immunogenicity are the major advantages over ADI-PEG. This again needs further confirmation in clinical trials.

Thirty years after the introduction of the first enzymatic drug, L-asparaginase, into the clinical arena for the treatment of lymphoid malignancies, we have now at least one, may be two, enzymatic drugs that have already proved capable of causing cancers to remit. This will no doubt rekindle the interests of nutritional manipulation and amino acid restriction in the treatment of cancer.

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