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Enzymes of creatine biosynthesis, arginine and methionine metabolism in normal and malignant cells

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The creatine/creatine kinase system decreases drastically in sarcoma. In the present study, an investigation of catalytic activities, western blot and mRNA expression unambiguously demonstrates the prominent expression of the creatine-synthesizing enzymes L-arginine:glycine amidinotransferase and *N*-guanidinoacetate methyltransferase in sarcoma, Ehrlich ascites carcinoma and Sarcoma 180 cells, whereas both enzymes were virtually undetectable in normal muscle. Compared to that of normal animals, these enzymes remained unaffected in the kidney or liver of sarcoma-bearing mice. High activity and expression of mitochondrial arginase II in sarcoma indicated increased ornithine formation. Slightly or moderately higher levels of ornithine, guanidinoacetate and creatinine were observed in sarcoma compared to muscle. Despite the intrinsically low level of creatine in Ehrlich ascites carcinoma and Sarcoma 180 cells, these cells could significantly take up and release creatine, suggesting a functional creatine transport, as verified by measuring mRNA levels of creatine transporter. Transcript levels of arginase II, ornithine-decarboxylase, *S*-adenosyl-homocysteine hydrolase and methionine-synthase were significantly upregulated in sarcoma and in Ehrlich ascites carcinoma and Sarcoma 180 cells. Overall, the enzymes related to creatine and arginine/methionine metabolism were found to be significantly upregulated in malignant cells. However, the low levels of creatine kinase in the same malignant cells do not appear to be sufficient for the building up of an effective creatine/phosphocreatine pool. Instead of supporting creatine biosynthesis, L-arginine:glycine amidinotransferase and *N*-guanidinoacetate methyltransferase appear to be geared to support cancer cell metabolism in the direction of polyamine and methionine synthesis because both these compounds are in high demand in proliferating cancer cells.

In a previous study concerning the status of the creatine/creatine kinase (CK) system in relation to sarcoma development, we demonstrated that creatine, phosphocreatine (PCr) and creatine kinase decreased progressively in sarcoma tissue compared to normal contralateral muscle [1]. Protein and mRNA expression levels of creatine kinase isoforms were significantly downregulated. From that study, it appeared that the creatine/PCr/CK system is gradually and steadily downregulated in sarcoma during tumor growth. Based on this finding, the question naturally arises as to the status of creatine transport and synthesis in

Abbreviations
3MC, 3-methylcholanthrene; AGAT, L-arginine:glycine amidinotransferase; CK, creatine kinase; CT-1, creatine transporter; EAC, Ehrlich ascites carcinoma; GAA, guanidinoacetic acid; GAMT, *N*-guanidinoacetate methyltransferase; ODC, ornithine decarboxylase; PCA, perchloric acid; PCr, phosphocreatine; S180, Sarcoma 180; SAH, *S*-adenosyl homocysteine; SAM, *S*-adenosyl methionine.

tumor cells or in tumor-bearing animals and how this may change during tumor progression. Interestingly, it was previously shown that Ehrlich ascites carcinoma (EAC) cells, a rapidly growing, highly dedifferentiated malignant cell line, can indeed transport creatine and cyclocreatine [2]. Moreover, these cells can phosphorylate a significant amount of creatine under favourable conditions, although the intrinsic CK activity in this type of cell is low, similar to the findings reported by our own laboratory [3].

Creatine is synthesized in a two-step process [4]. L-arginine:glycine amidinotransferase (AGAT; EC 2.1.4.1) is the first enzyme, prominently expressed in the kidney and pancreas [4], that catalyzes the transamidation of guanidine group from arginine to glycine, yielding guanidinoacetic acid (GAA) and ornithine (Fig. 1). GAA, thus formed, enters the circulation to reach the liver. Here, it is methylated by *N*-guanidinoacetate methyltransferase (GAMT; EC 2.1.1.2), which is prominently expressed in this organ to yield creatine. The methyl group donor is *S*-adenosyl methionine (SAM), which is subsequently converted to *S*-adenosyl homocysteine (SAH). Creatine then is somehow transported out of the liver to enter the blood circulation and reaches different creatine-requiring target tissues, such as muscle, brain and heart, etc., through an active Na^+/Cl^- dependent creatine transporter (CT-1) [4,5].

Besides being a precursor of creatine synthesis, arginine is additionally involved in several biosynthetic pathways that include a number of enzymes such as arginase (EC 3.5.3.1), arginine decarboxylase (EC 4.1.1.19) and nitric oxide synthase (EC 1.14.13.39) [6,7]. Cellular arginases play an important role in ammonia detoxification and urea synthesis and also provide ornithine for polyamine, glutamine and proline synthesis. In the mammalian liver, arginase I (a cytosolic enzyme) directs ornithine to polyamine synthesis due to its co-localization with ornithine decarboxylase (ODC; EC 4.1.1.17), a cytosolic enzyme. In extra-hepatic tissues, arginase II (a mitochondrial enzyme) is mainly involved in proline and glutamine synthesis owing to its co-localization with ornithine aminotransferase (EC 2.6.1.13), which is a mitochondrial enzyme. On the other hand, during the process of formation of creatine from GAA by GAMT, SAM is converted to SAH and the later is converted to homocysteine by the enzyme SAH hydrolase (EC 3.3.1.1). Methionine synthase (EC 2.1.1.13) converts homocysteine to methionine.

Tissue metabolism of arginine and methionine is of high importance for the effective regulation of cell death and survival in normal as well as in tumor cells. Polyamines also play an essential role in this respect [8]. There are reports that tumor cells accumulate polyamines in high concentrations [9,10]. Moreover, transmethylation reactions such as DNA methylation are highly prevalent in tumor cells [11–13]. Methionine serves as a precursor molecule for these transmethylation reactions, providing SAM as a methyl donor. In this context, ornithine and SAH, which are the byproducts of the AGAT and GAMT reaction, respectively, have immense importance as far as tumor metabolism is concerned.

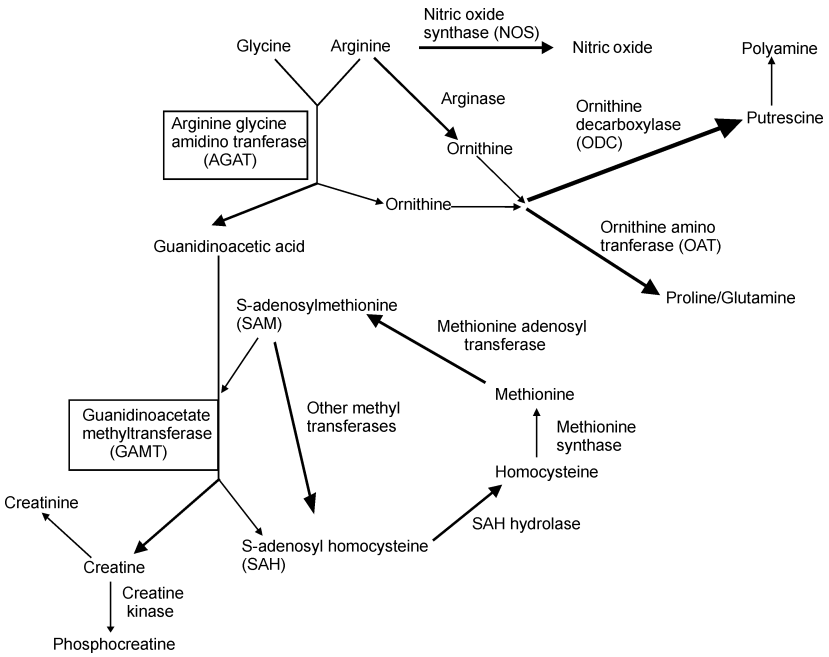

Fig. 1. Schematic diagram of creatine, arginine and methionine metabolism in mammalian tissues.

Some previous studies discretely revealed certain aspects of creatine synthesis and transport in either tumor-bearing subjects or in tumor cells [2,14,15]. However, there exists a gap concerning the status of creatine synthesis and transport and the role of the respective enzymes in tumor cell metabolism. Against this background, we studied creatine synthesizing enzymes, AGAT and GAMT, as well as CT-1 and actual creatine transport. The enzymes intimately linked with creatine biosynthesis were also studied. We conducted our studies in the solid sarcoma tissue of mice (induced with carcinogen in hind leg muscle) and compared the changes, if any, with the hind leg muscle taken from unaffected mice of the same age. Different parameters in the kidney, liver or sera of sarcoma-bearing and normal mice were also studied to ascertain the effect of tumor load in the overall metabolism of creatine and related metabolites in the animal. Similar studies were performed with EAC and S180 cells to confirm the tumor cell specificity of different alterations observed in sarcoma tissue. Cellular uptake and the release of creatine were studied only *in vitro* with EAC and S180 cells because *in vivo* studies with sarcoma tissue are difficult to perform.

Results

We directly measured the catalytic activity of the enzymes and the amount of relevant metabolites in relation to creatine metabolism. A parallel immunoblot and an mRNA expression study of the related enzymes were also performed. Creatine uptake and depletion in two model malignant cells were measured as well.

Catalytic activities of AGAT, GAMT and arginase II

Table 1 shows that activities of both AGAT and GAMT in sarcoma tissue were significantly higher compared to normal muscle, where it was almost undetectable. Table 1 also shows that the activities of these two enzymes in the three tumors (EAC, S180 and sarcoma tissue) were quantitatively more or less similar. Arginase II activity was also quite high in these three tumors. On the other hand, the activities of these three enzymes remained unaltered in tumor-bearing mice kidney or liver compared to that of tumor-free mice.

Table 1. Specific activities of AGAT, GAMT and arginase II from different normal and tumor sources. Values are the mean ± SD ($n = 3$ per group). Specific activity is expressed as nmol·60 min^{-1}·mg^{-1} protein. ND, not detectable; NM, not measured.

	AGAT	GAMT	Arginase II
Normal mice muscle	ND	ND	ND
Sarcoma (3MC)	25.0 ± 5.6	36.7 ± 5.6	49.1 ± 1.3
Normal mice kidney	59.2 ± 5.7	NM	190.5 ± 3.5
Sarcoma-bearing mice kidney	58.0 ± 3.6	NM	189.6 ± 1.6
Normal mice liver	NM	36.7 ± 0.6	NM
Sarcoma-bearing mice liver	NM	32.5 ± 3.5	NM
EAC	24.8 ± 1.1	34.3 ± 1.3	42.1 ± 0.8
S180	29.1 ± 0.5	31.8 ± 2.5	74.5 ± 1.3

Estimation of ornithine and GAA

The considerable and significant activities of AGAT and arginase II in the three types of tumor cells prompted us to measure the level of GAA and ornithine in tumor cells. Table 2 shows that the level of ornithine was quite high in EAC and S180 cells. In sarcoma, it was comparable to the level in the kidney but significantly higher than the level in normal muscle. In sarcoma, the level of ornithine was comparable to that of the kidney but significantly higher than that of normal muscle. The GAA content in sarcoma tissue was also significantly higher compared to that of normal muscle. The tissue contents of both these metabolites remained almost unaltered in the kidney of tumor-bearing mice. Ornithine contents in the sera of tumor-bearing mice and tumor-free mice showed no differences, whereas that of GAA showed significant differences (Table 2).

Estimation of creatine and creatinine

We previously observed in the sarcoma tissue of mice that the creatine content is very low compared to that of normal muscle [1]. In the present study, we observed that the creatine content was also very low in EAC

Table 2. Ornithine, GAA, creatine and creatinine contents from different normal and tumor sources. Values are expressed as μg·mg^{-1} protein in case of tissues and as μg·mL^{-1} in the case of sera. Values are the mean ± SD. $^{**}P < 0.001$ versus sarcoma; $^{*}P < 0.05$ versus normal muscle ($n = 3$ per group). NM, not measured.

	Ornithine	GAA	Creatine	Creatinine
Normal mice muscle	2.0 ± 0.4	11.4 ± 1.7	72.6 ± 2.0	0.54 ± 0.5
Sarcoma (3MC)	3.2 ± 0.3*	18.0 ± 2.3*	7.2 ± 0.4**	0.74 ± 0.3
Normal mice kidney	3.5 ± 0.7	24.0 ± 1.4	NM	NM
Sarcoma-bearing mice kidney	4.0 ± 1.4	25.0 ± 1.4	NM	NM
EAC	5.05 ± 0.1	10.15 ± 2.6	11.4 ± 0.8	0.55 ± 0.07
S180	6.3 ± 1.8	10.5 ± 2.1	8 ± 1.4	0.33 ± 0.19
Normal mice sera	112.7 ± 3.1	156.0 ± 5.7	21.6 ± 5.7	8.6 ± 0.6
Sarcoma-bearing mice sera	116.7 ± 3.2	236 ± 3.9	24.5 ± 0.7	10.5 ± 0.7

and S180 cells and that these concentrations were similar to those of sarcoma tissue. However, there was no significant difference in creatinine content between normal muscle and sarcoma tissue, EAC and S180 cells. The creatine and creatinine contents in the sera of tumor-bearing mice and tumor-free mice also showed little or no difference (Table 2).

Western blot analysis

The results presented in Table 1 show significant catalytic activity of both AGAT and GAMT in sarcoma tissue compared to that of normal muscle, where the activities of these enzymes were almost undetectable. Therefore, we additionally performed immunoblot experiments with antibodies raised against rat AGAT and GAMT proteins. Figure 2A,B show that, using these antibodies, it was possible to detect both of these enzymes in sarcoma tissue as well as in EAC and S180 cells, which is in agreement with the presence of significant catalytic activities of these enzymes in all three malignant sources, as noted above. Figure 2A also shows that the AGAT protein level was almost identical in sarcoma tissue and in the kidneys of both normal and tumor-bearing mice, whereas the AGAT levels were lowest in normal muscle and intermediate in EAC and S180 cells. Figure 2B shows that the GAMT levels were more or less similar in all three tumor samples and also in the liver, but remained undetectable with this method in normal muscle.

RT-PCR and mRNA expression analysis of AGAT and GAMT

Both measurements of catalytic activity and immunoblot experiments showed a significant increase in the enzymatic activity and protein expression of both AGAT and GAMT in sarcoma and in two other malignant cell lines compared to the levels of these two creatine synthesizing enzymes in normal muscle. Thus, to determine whether this up-regulation is taking place at the transcriptional level, we measured and compared the expression of mRNA of these enzymes in normal and malignant cells. Figure 2A shows an almost equally elevated and high expression of AGAT mRNA in all three tumor samples, whereas it is almost undetectable in normal muscle. GAMT mRNA expression was almost equal in all three tumor samples (Fig. 2B), whereas, in normal muscle, this expression was very low. In the kidney and liver, mRNA expression of the respective enzymes remained unchanged in both tumor-bearing and tumor-free mice. Overall, these results are in agreement with the results of enzymatic assays and immunoblot experiments, and suggest that the increase in AGAT and GAMT in malignant cells is due to the increased mRNA synthesis and/or increased stability of the synthesized mRNAs.

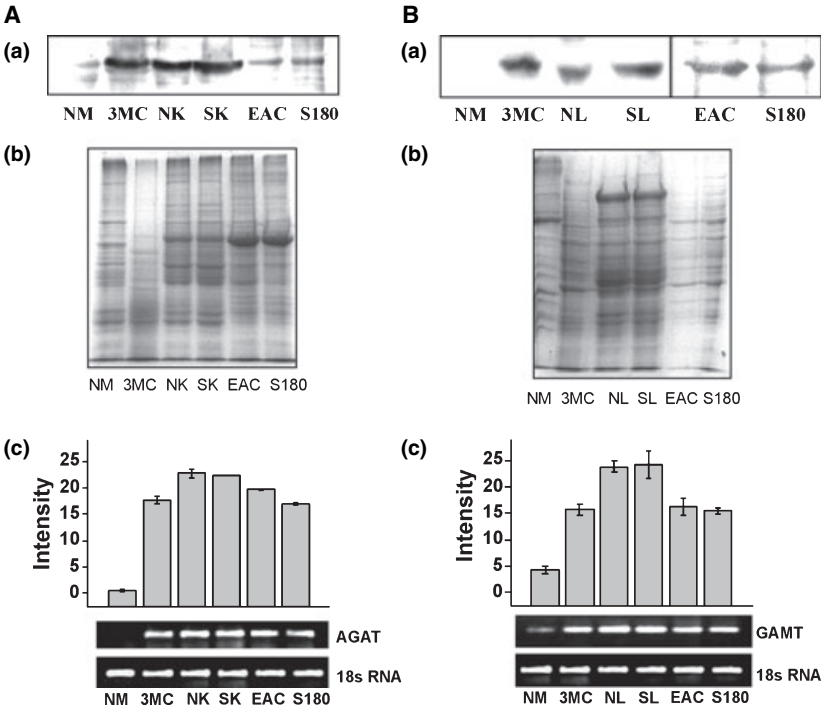

Fig. 2. Immunoblot and mRNA expression of (A) AGAT and (B) GAMT: (a) immunoblot; (b) parallel gels stained with Coomassie blue to confirm equal protein loading; and (c) densitometric analysis of the amplified PCR fragments (mean ± SD; $n = 3$ per group) and representative agarose gel of the amplified DNA fragments. NM, normal muscle; 3MC, sarcoma tissue; NK, normal mice kidney; SK, sarcoma-bearing mice kidney; NL, normal mice liver; SL, sarcoma-bearing mice liver. In (a) and (b), the protein loaded on each lane was 25 μg.

Creatine uptake and release study in EAC and S180 cells and creatine transporter mRNA expression

The significant presence of both of the enzymes responsible for creatine biosynthesis, AGAT and GAMT, in all three types of malignant cell lines suggests the possible presence of creatine in these cells. However, as noted above, the intrinsic level of creatine itself is very low in these cells. Thus, we studied the uptake and release of creatine in EAC and S180 cells as a model system. Figure 3 shows that, if the cells were incubated for 1 h in the uptake medium containing creatine, both cell types accumulated significant amounts of creatine. On the other hand, when these creatine-loaded cells were placed in creatine-free medium, their creatine contents were depleted with time. From these experiments, it is obvious that, under favourable conditions, creatine can be transported into and out of these cells, thus being moved both ways, by the tumor cells.

We also undertook CT-1 mRNA expression studies in sarcoma tissue as well as in EAC and S180 cells. The results show that the mRNA expression is very similar in all three malignant cell types and also rather similar to that in normal muscle (Fig. 3).

mRNA expressions of some related enzymes

Furthermore, we studied mRNA expression of different enzymes involved in arginine and methionine metabolism because these enzymes are intimately related to creatine biosynthesis (Fig. 4). Between the two isoforms of cellular arginases, only the mitochondrial arginase (arginase II) shows significant expression in tumor models. ODC mRNA is also significantly high in tumor cells, indicating the activation of polyamine biosynthesis from ornithine produced by AGAT and arginase II. In these tumor cells, SAH hydrolase and methionine synthase mRNA levels are high, indicating the activation of the pathway for the utilization of SAH and formation of methionine in tumor cells.

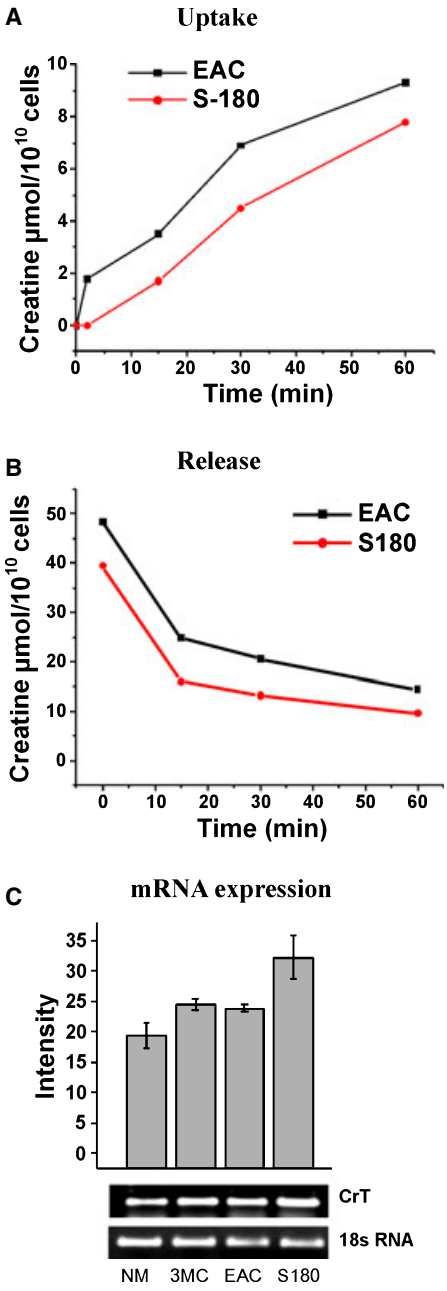

Fig. 3. Creatine uptake (A) and release (B) as measured *in vitro* with EAC and S180 cells and mRNA expression of creatine transporter (C) by densitometric analysis of the amplified PCR fragments (mean ± SD; $n = 3$ per group). NM, normal muscle; 3MC, sarcoma tissue.

Discussion

In the present study, we investigated the comparative status of creatine biosynthesis in normal muscle and sarcoma tissue and also in those organs of normal and sarcoma-bearing mice that are primarily involved in the biosynthesis of this metabolite.

AGAT, a mitochondrial enzyme, is highly expressed in the mammalian kidney and pancreas, but the enzyme, albeit at significantly lower levels, can also be found in the brain, heart, lung, muscle, spleen and testes, etc. [4,16]. AGAT is bound to the mitochondrial inner membrane and competes with arginases for the same intracellular pool for arginine. On the other hand, AGAT is also the rate-limiting enzyme of creatine biosynthesis and the enzyme is subject to end-product repression by ornithine and creatine [17–19]. Thus,

AGAT is involved in arginine-related, as well as in creatine metabolism. On the other hand, a high expression of GAMT had been found in the liver and pancreas and other tissues, such as the testes, epididymis and ovary, whereas the expression of both enzymes has been reported to be low in skeletal muscle [4]. However, the status of these enzymes had not been previously studied in sarcoma tissue. In the present study, we show that, upon malignant transformation, the skeletal muscle of mice showed a prominent up-regulation of the expression and enzymatic activity of both AGAT and GAMT. The specific activity of AGAT in sarcoma tissue reached almost 50% of that observed in the kidney and it was also observed that the tumor load had no significant effect on the AGAT activity of the kidneys of tumor-bearing mice (Table 1). Although some previous studies reported a reduction in AGAT activity in the kidneys of tumor-bearing mice and the rat [14,15], we only found a statistically insignificant difference of AGAT between the kidneys of tumor-bearing and tumor-free mice. Similarly, the specific activity of GAMT in sarcoma tissue was found to be almost equal to that of the liver. No change in GAMT activity was observed in the liver due to tumor load. Both AGAT and GAMT were also highly detectable in EAC and S180 cells and the values were similar to those of sarcoma tissue. All these results were confirmed by immunoblotting as well as by mRNA expression studies. The catalytic activities, immunoblotting and mRNA expression studies of AGAT and GAMT were in agreement with a significant up-regulation of both enzymes in sarcoma tissue, as well as in two model malignant cell lines.

Interestingly, despite the high activities of these creatine-synthesizing enzymes, creatine content was found to be very low in sarcoma tissue [1] and also in EAC cells [3], as had been previously found in our laboratory. One possible explanation for this finding is that

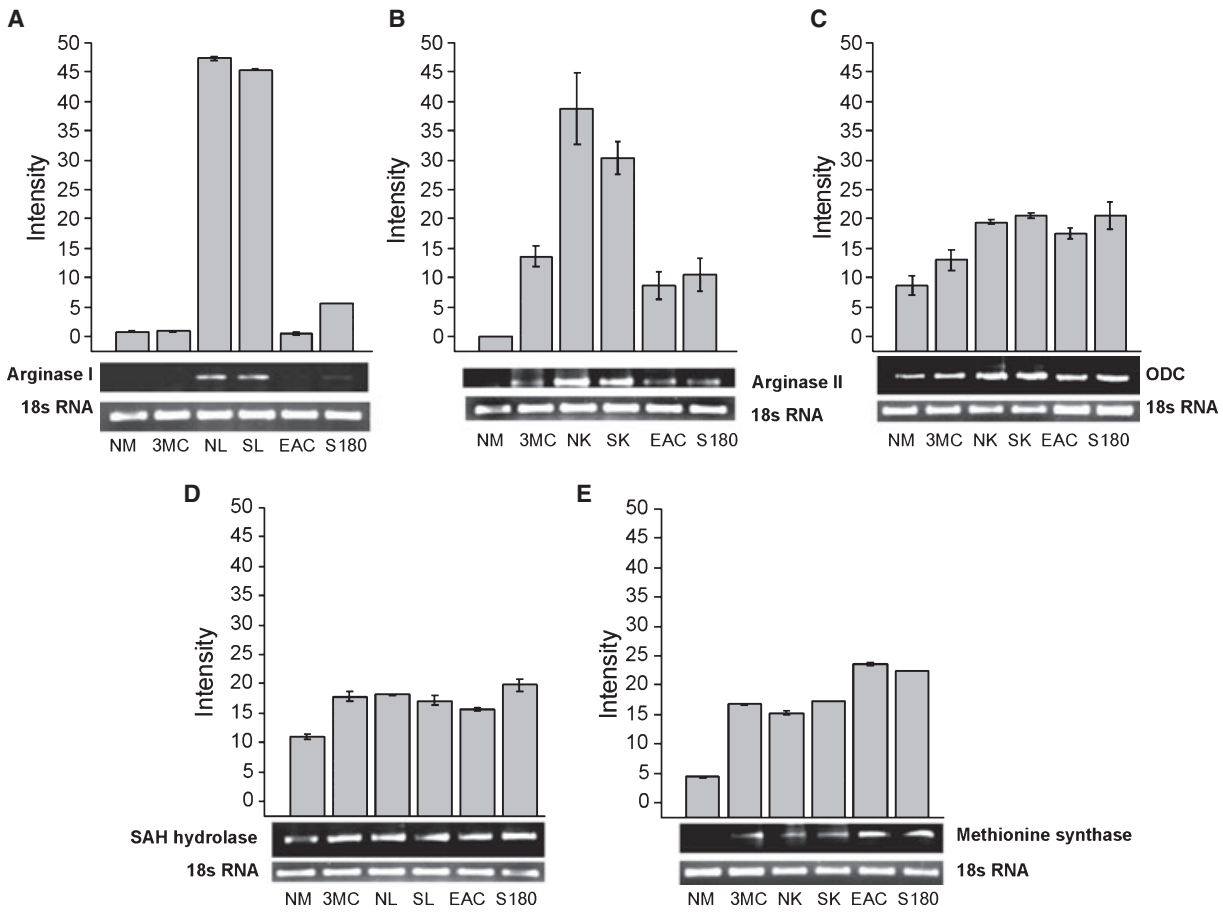

Fig. 4. mRNA expression of (A) arginase I, (B) arginase II, (C) ornithine decarboxylase, (D) SAH hydrolase and (E) methionine synthase. Densitometric analysis of the amplified PCR fragments (mean ± SD; $n = 3$ per group) and representative agarose gel of the amplified DNA fragments. NM, normal muscle; 3MC, sarcoma tissue; NK, normal mice kidney; SK, sarcoma-bearing mice kidney; NL, normal mice liver; SL, sarcoma-bearing mice liver.

the tumor tissue itself acts as a creatine synthesizing organ, with both of the enzymes, AGAT and GAMT, being expressed at fairly high concentrations. However, with a very low total CK activity [1,3], no effective pool of PCr could be built up in these tissues. It appears that, in contrast to that of normal muscle cells, the plasma membrane of tumor cells has an built-in mechanism for the export of creatine, possibly related to the postulated creatine exporter in liver. In this respect, it is also worth noting that thyroid hormones are known to regulate total CK activity as well as creatine transport [20,21]. In hypothyroidism, there was a decrease in total CK activity, whereas, on administration of thyroxine, there were remarkable changes in creatine transport in cardiac cells. A similar phenomenon of decreased CK activity and/or increased permeability of creatine against its concentration gradient across the membrane occurs in malignant cells, and needs further investigation. The presence of both AGAT and GAMT in EAC and S180 cells and in sarcoma tissue indicates that the upregulated proteins are entirely tumor-cell specific.

On the other hand, the influx and efflux rate of creatine in both EAC and S180 cells shows that the tumor cells became highly permeable to this metabolite. However, the level of circulating creatine in tumor-bearing mice blood did not differ significantly from that of tumor-free mice blood. Hence, the metabolic fate of creatine that is being synthesized by tumor cells could not be determined precisely. Furthermore, there were no significant differences in ornithine and creatinine content in the blood of tumor-bearing mice compared to that in tumor-free mice, with the values of GAA differing significantly, being higher in tumor-bearing mice (Table 2). The latter could be due to the higher levels of AGAT (with GAA as the product) in tumor-bearing mice. To analyze the significance of AGAT and GAMT expression in tumor cells, we investigated whether ornithine and SAH production would be the major aim for the upregulation of these two enzymes in tumors.

As noted earlier, owing to their co-localization, the cytosolic enzymes, arginase I and ODC direct ornithine towards polyamine synthesis and the mitochondrial enzymes arginase II and ornithine aminotransferase favor the channeling of ornithine to proline/glutamine synthesis. There are several reports of elevated arginase activity [22,23] in tumors. Ornithine has several metabolic fates (Fig. 1). The most important one in tumor cells is the conversion of ornithine to putrescine, which is the precursor molecule of polyamines such as spermidine and spermine. This reaction is catalyzed by ornithine decarboxylase and the enzyme was found to be increased in several forms of human and rodent tumors [24,25]. Increased polyamine levels have been reported in a large number of tumors [8–10,26–30].

Our observations indicated that ornithine content and ODC expression were high in tumors. This indicates that the pathway of polyamine formation from arginine metabolism is favored in this tissue. Again, arginase II, which is a mitochondrial enzyme, is highly expressed in tumors, whereas cytosolic arginase I expression is negligible in this tissue. It is possible that arginine is catabolyzed to ornithine via the mitochondrial enzymes, AGAT and arginase II, more efficiently than by its cytosolic counterpart, arginase I. Therefore, AGAT, could be playing a dual role by: (a) providing GAA as a substrate for GAMT and (b) providing ornithine to ODC for polyamine synthesis. Moreover, it was found that AGAT activity was repressed by ornithine [17]. The high ODC content, as described in our study, might be responsible for the effective removal of ornithine from the vicinity of AGAT, thereby protecting against the possible suppression of its activity.

On the other hand, GAMT activity is strongly regulated by the SAH concentration. An increased SAH level or inhibition of SAH hydrolase was found to inhibit GAMT activity [31]. There are some reports of decreased GAMT levels in rat liver with induced hepatocarcinoma [32,33]. Decreased levels of SAM [32] and an increased level of creatine [33] have also been reported in these studies. SAM is an important metabolite, which acts as precursor molecule for polyamine formation (aminopropylation), and functions as the sole methyl group donor in various other transmethylation reactions, as needed for creatine and glutathione synthesis (transulfuration) [34–36]. Each transmethylation reaction yields SAH that is further converted into homocyst(e)ine by SAH hydrolase. In normal cells, homocyst(e)ine is remethylated to methionine via either of the two enzymes methionine synthase and/or betaine-homocysteine methyltransferase (EC 2.1.1.5). Interestingly, methionine auxotrophy had been proposed as one of the major phenotypic expressions of a diverse type of tumor cells [37]. Methionine dependency was explained by the increased rate of transmethylation reactions in transformed cells, whereas the methionine synthase level remained unaltered [37]. However, there are conflicting reports about the status of methionine synthase in tumor cells, with some studies showing it to be defective, whereas others find it to be unaltered in tumors [38]. In spite of this anomaly, a general conclusion would be that a tumor demands a surplus amount of methionine that could be either synthesized from homocyst(e)ine or obtained from the host tissue.

We have observed that the mRNA expression of both SAH hydrolase and methionine synthase increased in sarcoma tissue and also in EAC and S180 cells (Fig. 4). However, there was no effect of tumor load on the transcript levels in the kidney or liver tissue. The findings suggest the possibility that SAH produced from the GAMT reaction could be recycled through SAH hydrolase and methionine synthase to homocyst(e)ine and methionine (Fig. 1).

It appears that the inclusion of AGAT and GAMT into the metabolic pathway of arginine and methionine, respectively, could definitely provide extra advantages to tumor cells. These will help to promote ornithine and SAH production, which in turn could be used for the formation of polyamines and methionine. An investigation of the level of different intermediate metabolites and the expression of different enzymes in the metabolic cycle of arginine and methionine further strengthens this view.

Estrogen administration increases the expression of AGAT in chick liver, indicating that AGAT may be a target of the estrogen receptor [39]. Estrogens and estrogen receptors are considered among the major effectors of carcinogenesis in several forms of human and rodent tumor [40,41]. On the other hand, no systematic study was conducted to demonstrate the transcriptional regulation of GAMT. Extensive research is needed to determine the regulatory factors modulating AGAT and GAMT over-expression and their significance in tumor cell metabolism. If correct, these creatine synthesizing enzymes could possibly be considered as targets for cancer therapy. Interestingly, in this connection, creatine supplementation that is known to downregulate AGAT [19] was shown to exert quite potent anti-cancer action in several cell culture and animal models [42,43].

Experimental procedures

Creatine, ornithine, GAA, GSH, SAM, hydrindantin hydrate, 3-methylcholanthrene (3MC), nitrocellulose membrane (0.45 μM pore size) and anti-(rabbit IgG) (whole molecule) peroxidase conjugated were obtained from Sigma Chemical Co. (St Louis, MO, USA). Luminol reagent was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). M-MLVRT, Taq polymerase, dNTP, random hexamer and Trizol reagent were from Invitrogen (Carlsbad, CA, USA). Creatine kinase assay and creatinine estimation kits were obtained from Bayer Diagnostics India (Baroda, India). Other chemicals were of analytical grade and obtained from local manufacturers.

Growth of tumors

Animal experiments were carried out in accordance with the guidelines of the institutional ethics committee for animal experiments. Appropriate measures were taken to minimize pain or discomfort for animals.

EAC and S180 cells were grown intra-peritoneally in sexually mature Swiss albino female mice. Sarcoma was induced with 3MC in one hind leg of Swiss albino female mice as described previously [1]. EAC and S180 cells were collected in normal saline (0.9% NaCl) from the intra-peritoneal cavity of mice, washed with 0.45% NaCl until it was free of red blood cells and finally suspended in normal saline. A full-grown sarcoma tissue, as confirmed by histological examination [1], was excised from the mice hind leg and immediately placed in ice-cold buffer. Skeletal muscle from the hind leg, kidney and liver were excised soon after sacrificing the mice and immediately transferred to ice-cold buffer.

Metabolite estimations

If not mentioned otherwise, EAC and S180 cells were quickly homogenized in four volumes, and tissues in six volumes, of ice-cold $NaPO_4$ buffer (25 mM, pH 7.4). The homogenate was made protein free by immediately adding 5% ice-cold perchloric acid (PCA) and the PCA was neutralized with KOH solution. By this way, the *in vivo* concentrations of cell metabolites of interest were stabilized. Blood from normal and sarcoma-bearing mice was collected and the sera were also made protein free with 5% cold PCA and neutralized as before. Different metabolites were determined in this neutralized protein-free extract. Ornithine was estimated according to Chinard *et al.* [44], GAA by the modified Sakaguchi reaction [45] and creatine by α-naphthol-diacetyl [46]. Creatinine was estimated by a creatinine estimation kit based on picric acid and NaOH. These metabolites were estimated from normal muscle, sarcoma tissue, sera of normal and sarcoma-bearing mice, as well as from EAC and S180 cells. In addition, ornithine and GAA were estimated also from the kidney of normal and sarcoma-bearing mice.

Enzyme assay

AGAT and mitochondrial arginase (arginase II) were assayed in mitochondrial preparations of EAC and S180 cells, mice muscle, sarcoma tissue and the kidney of normal and sarcoma-bearing mice. Mitochondria from EAC and S180 cells were prepared according to Moreadith and Fiskum [47] and from normal muscle and sarcoma tissue as described previously [1]. Kidney mitochondria were prepared according to Magri *et al.* [48]. AGAT and arginase II were assayed by incubating mitochondrial

preparations in 50 mM $NaPO_4$ buffer (pH 7.4) at 37 °C for 1 h. For AGAT assay, 2.5 mM arginine and 5 mM glycine were added to incubation medium and, for the arginase II assay, only 2.5 mM arginine was used. The reaction was stopped with 5% ice-cold PCA to denature the protein, neutralized with KOH solution and ornithine was estimated.

The GAMT assay was performed according to Cantoni *et al.* [49] with minor modifications. Briefly, minced muscle, sarcoma or liver tissues and EAC and S180 cells were homogenized in six volumes of 100 mM Na-acetate buffer (pH 5.0) and centrifuged at 10 000 ***g*** for 10 min. The supernatant was collected and subjected to ammonium sulfate fractionation. The protein that precipitated at 25–40% saturation of ammonium sulfate was collected and dissolved in a minimum volume Na-acetate buffer (100 mM, pH 5.0). GAMT was assayed by incubating this protein precipitate in a solution containing 50 mM Tris–Cl (pH 7.4), 0.5 mM SAM, 3 mM GAA and 8 mM GSH at 37 °C for 1 h. The reaction was stopped with 5% trichloroacetic acid and centrifuged at 15 000 ***g*** to remove protein precipitates. The clear supernatant was autoclaved for 20 min to convert entire creatine produced to creatinine, which was estimated as described earlier.

Creatine uptake and depletion study

The creatine uptake study in EAC and S180 cells was performed according to Annesley *et al.* [2] with minor modifications. The cells were incubated with 5 mM creatine in incubation buffer (50 mM Hepes, 80 mM NaCl, 10 mM Na_2HPO_4, 8 mM KCl, 1.5 mM $MgSO_4$, 1 mv $CaCl_2$, 1% BSA and 20 mM glucose, pH 7.4) at 37 °C for 1 h and the uptake was studied at the indicated time points. After 1 h, the cells were washed twice in wash buffer (130 mM NaCl, 8 mM KCl, 1.5 mM $MgSO_4$, 10 mM Na_2HPO_4, 1 mM $CaCl_2$ and 5.5 mM glucose) and suspended in a fresh incubation buffer, but this time without creatine. Creatine content within the cells was monitored at indicated time points to ascertain the creatine depletion rate. To measure creatine content, cells were collected by centrifugation at 1000 ***g*** for 5 min and washed in wash buffer and sonicated to disrupt the cells. Ice-cold 5% PCA was added to precipitate the proteins, which was removed by centrifugation and the supernatant was neutralized with KOH. Creatine was estimated in this neutralized solution.

All operations requiring protein precipitation and neutralization were perfomed when keeping the samples on ice.

Immunoblot

Immunoblot was performed as mentioned by Patra *et al.* [1]. Briefly, for AGAT, mitochondrial protein from different normal and tumor sources were used for immunoblot. For GAMT, normal and tumor tissue or cells were homogenized in six volumes of 50 mM Tris–Cl buffer (pH 7.4) containing 150 mM NaCl and 0.1% Triton X-100. The homogenate was incubated at 4 °C for 15 min and centrifuged at 10 000 ***g*** for 10 min. The supernatant was collected and used for western blotting. Primary antibody dilutions used for immunoblot were: AGAT (1 : 2500) and GAMT (1 : 1000). Secondary antibody dilution was 1 : 5000 anti-(rabbit peroxidase-conjugated IgG) for both AGAT and GAMT. Equal protein loading was confirmed with a parallel gel stained with Coomassie blue.

mRNA expression study

Total cellular RNA of EAC and S180 cells, normal muscle, sarcoma tissue, kidney and liver were isolated with Trizol reagent as per the manufacturer's instructions. AGAT, GAMT, CT-1, arginase I and II, ODC, SAH hydrolase and methionine synthase expressions were quantified by RT-PCR. 18S RNA was chosen as the housekeeping gene for normalization because its expression did not differ between the different types of tissues. Primer sequences for different enzymes are given in Table 3. The reaction cycles of PCR were performed in the range that demonstrated a linear correlation between the amount of cDNA and the yield of PCR products. PCR amplified DNA fragments were run on a 1.5% agarose gel stained with ethidium bromide and visualized and photographed by irradiating with UV light. The band intensities were calculated with QUANTITY ONE 1-D analysis software (Bio-Rad, Hercules, CA, USA).

Table 3. Primers used for PCR amplification.

Gene	Forward primer Reverse primer (5′- to 3′)	PCR product size
AGAT	ATG GAA GGA GTG ACC GTG AG GGC ACC ACG ATG GAA GTA GT	203
GAMT	GGC AGC CAC ATA AGG TTG TT CGT GAG GTT GCA GTA GGT GA	211
Creatine transporter	GAA ATG GTG CTG GTC CTT CTT CAC GTC ACA TGA CAC TCT CCA CCA CGA	353
Arginase I	GTG AAG AAC CCA CGG TCT GT CTG GTT GTC AGG GGA GTG TT	209
Arginase II	GGA TCC AGA AGG TGA TGG AA AGA GCT GAC AGC AAC CCT GT	199
ODC [50]	CAG CAG GCT TCT CTT GGA AC CAT GCA TTT CAG GCA GGT TA	602
SAH hydrolase [51]	CTG AGG AGA CCA CGA CTG TGC CCA CAT CAC CAT AGC	216
Methionine synthase	CAT CCA AGA GTG TGG TGG TG ATA AAC GTG GGC TTC ACT GG	211
18S RNA	CAC GGC CGG TAC AGT GAA AC CCC GTC GGC ATG TAT TAG CT	165

Protein estimation

Protein estimation was performed with BSA as a standard by the method of Lowry, as outlined by Layne [52].

Statistical analysis

Data are presented as the means ± SD for n separate animals. In the figures, vertical bars, which represent the SD, are absent if smaller than the symbol. A comparison between different experimental groups was conducted using Student's two-tailed t-test.

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