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ORIGINAL ARTICLE

A short review on creatine–creatine kinase system in relation to cancer and some experimental results on creatine as adjuvant in cancer therapy

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Abstract The creatine/creatine kinase (CK) system plays a key role in cellular energy buffering and transport. In vertebrates, CK has four isoforms expressed in a tissue-specific manner. In the process of creatine biosynthesis several other important metabolites are formed. The anticancer effect of creatine had been reported in the past, and recent literature has reported low creatine content in several types of malignant cells. Furthermore, creatine can protect cardiac mitochondria from the deleterious effects of some anticancer compounds. Previous work from our laboratory showed progressive decrease of phosphocreatine, creatine and CK upon transformation of skeletal muscle into sarcoma. It was convincingly demonstrated that prominent expression of creatine-synthesizing enzymes L-arginine: glycine amidinotransferase and *N*-guanidinoacetate methyltransferase occurs in sarcoma, Ehrlich ascites carcinoma and sarcoma 180 cells; whereas, both these enzymes are virtually undetectable in skeletal muscle. Creatine transporter also remained unaltered in malignant cells. The anticancer effect of methylglyoxal had been known for a long time. The present work shows that this anticancer effect of methylglyoxal is significantly augmented in presence of creatine. On creatine supplementation the effect of methylglyoxal plus ascorbic acid was further augmented and there was no visible sign of tumor. Moreover, creatine and CK, which were very low in sarcoma tissue, were significantly elevated with the concomitant regression of tumor.

Keywords Ascorbic acid · Cancer · Creatine · Creatine kinase · Methylglyoxal

Abbreviations

AGAT	L-Arginine: glycine amidinotransferase
CK	Creatine kinase
EAC	Ehrlich ascites carcinoma
GAMT	*N*-guanidinoacetate methyltransferase
MCK	Muscle-specific cytosolic CK
BCK	Brain-specific cytosolic CK
MitCK	Mitochondrial CK
sMitCK	Sarcomeric MitCK
uMitCK	Ubiquitous MitCK
MPT	Mitochondrial permeability transition

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Introduction

The creatine/creatine kinase system

The creatine/creatine kinase (CK)/phosphocreatine system plays a key role in cellular energy buffering and energy transport, particularly in cells with high and fluctuating

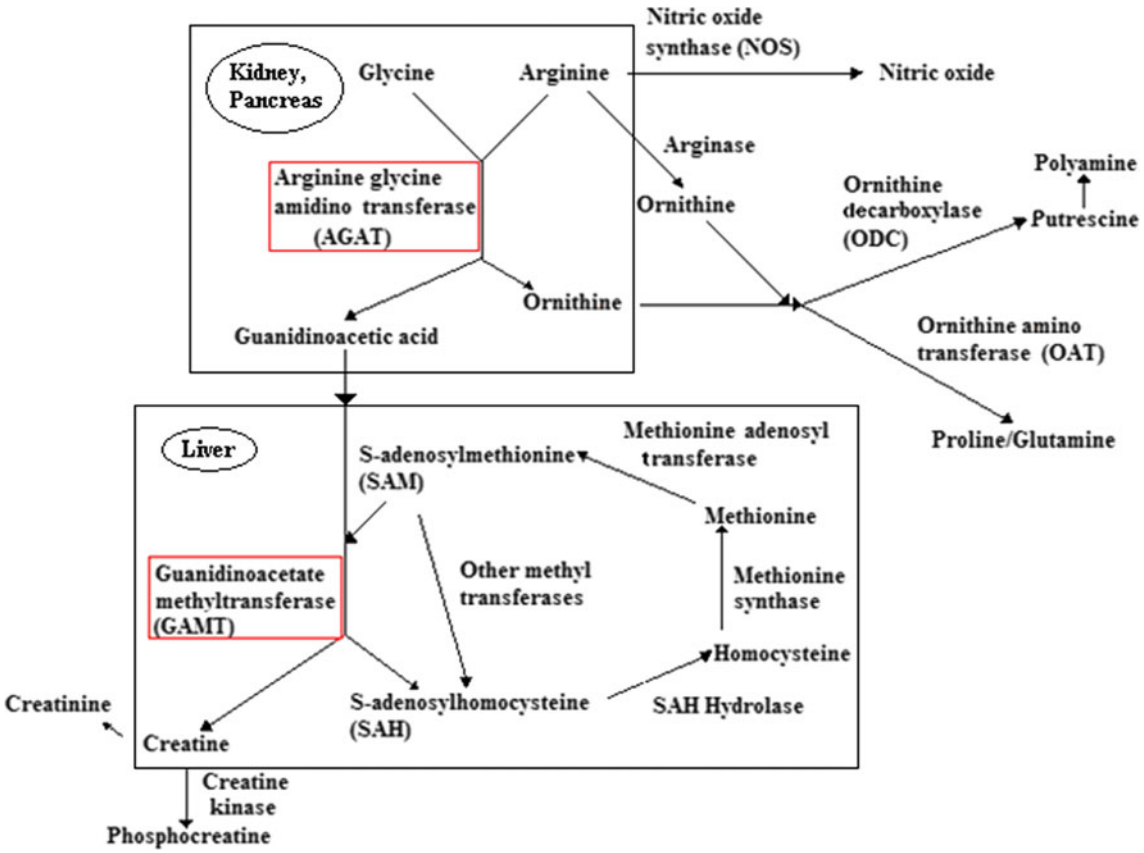

Fig. 1 Schematic presentation of creatine metabolism in mammalian tissues

energy requirements such as in skeletal and cardiac muscle, brain, photoreceptor cells, spermatozoa and electrocytes. The reversible transphosphorylation of creatine by ATP is catalyzed by the enzyme CK (EC 2.7.3.2).

$$\text{ADP} + \text{phosphocreatine} \leftrightarrow \text{ATP} + \text{creatine}$$

In vertebrates, four different CK isoforms are expressed in a tissue-specific manner. Muscle-specific cytosolic (MCK) is expressed specifically in sarcomeric skeletal and cardiac muscles, brain-specific cytosolic (BCK) is mainly expressed in brain, neuronal tissues and other non-muscle and non-cardiac tissues. In addition, there are two mitochondrial (MitCK) isoforms. Sarcomeric MitCK (sMitCK) is co-expressed with MCK in striated skeletal and heart muscles and ubiquitous MitCK (uMitCK) is present in smooth muscle, brain, neuronal and other non-muscle tissues and is co-expressed with BCK (for review Wyss and Kaddurah-Daouk 2000; Wallimann et al. 2011).

Creatine is synthesized in a two-step process. L-arginine: glycine amidinotransferase (AGAT; EC 2.1.4.1) is the first enzyme; prominently expressed in kidney and pancreas that catalyzes the transamidation of guanidine group from arginine to glycine yielding guanidinoacetic acid and ornithine; guanidinoacetic acid, thus formed, enters the circulation to reach the liver. Here, it is methylated by *N*-guanidinoacetate methyltransferase (GAMT; EC 2.1.1.2) that is prominently expressed in this organ to yield creatine. The methyl group donor is *S*-adenosyl methionine, which is subsequently converted to *S*-adenosyl homocysteine. Creatine is then transported out of the liver to enter the blood circulation and reaches different creatine-requiring target tissues, such as muscle, brain, heart, etc., through an active Na^+/Cl^- dependent creatine transporter (Wyss and Kaddurah-Daouk 2000; Bera et al. 2008). Thus, a significant proportion of the body's creatine is synthesized endogenously via a renal-hepatic axis, but the importance of these enzymes involved in creatine biosynthesis that are also expressed in other tissues, besides in kidney and liver, remains to be elucidated (Fig. 1).

Arginine is also involved additionally in several biosynthetic pathways by providing ornithine for polyamine, glutamine and proline synthesis. Moreover, during the process of formation of creatine from guanidinoacetic acid by GAMT, methionine is formed through *S*-adenosyl homocysteine (Morris 2009). Arginine, methionine and polyamines play essential roles in both survival and death of normal and tumor cells (Wallace 2009; Palmer and

Wallace 2010). Tumor cells accumulate polyamines in high concentrations (Pegg 1988). Moreover, methionine serves as a precursor in DNA methylation, which is highly prevalent in tumor cells (Jones and Gonzalgo 1997; Gilbert 2009). Methionine serves as a precursor molecule for these transmethylation reactions providing *S*-adenosyl methionine as a methyl donor (Jones and Gonzalgo 1997; Robertson 2001; Brosnan and Brosnan 2006). In this way, the byproducts of AGAT and GAMT reactions have immense importance in tumor metabolism and may have implications in the use of creatine as a therapy for cancer.

Creatine/CK system in relation to malignancy

As the creatine–CK system is possibly related with tumor metabolism through regulation of ATP production and/or modulation, the field of creatine–CK system in relation to malignancy remained under intense investigation. The anticancer effect of creatine and its analog cyclocreatine had been known for quite sometime (Miller et al. 1993; Lillie et al. 1993).

Growth rate inhibition of subcutaneously implanted tumors such as rat mammary tumors, sarcoma and human neuroblastoma cells in rat or in nude mice was observed when the animal's diet contained either creatine or cyclocreatine (Miller et al. 1993). Cyclocreatine inhibited the proliferation of L1236, a Hodgkin-disease derived cell line (Kornacker et al. 2001). Moreover, cyclocreatine had a unique mechanism of anticancer activity and is well-tolerated by cancer patients (Wyss and Kaddurah-Daouk 2000). Interestingly normal cell lines that express high CK level were not inhibited by cyclocreatine (Martin et al. 1994). We had also observed an augmentation of the anticancer effect of methylglyoxal and ascorbic acid by creatine (Ghosh et al. 2006).

The current data on creatine content, CK expression and the activities of different isoforms of CK in malignant cells and tumor-bearing animals, however, provides a somewhat ambiguous picture. Higher activities of different CK isoforms had been reported in malignancies (Gazdar et al. 1981; Zarghami et al. 1996; Meffert et al. 2005; Balasubramani et al. 2006). In contrast, there were also reports of decreased level of creatine and increased choline/creatine ratio and decrease in the activity of CK and some of its isoforms in several different forms of cancer (Tsung 1983; Joseph et al. 1997; Horska et al. 2001; Lehnhardt et al. 2005; Onda et al 2006; Panigrahy et al. 2006; Davies et al. 2008; Martínez-Bisbal and Celda 2009). Consequently, it was evident from the existing previous literature that a systematic study on creatine–CK system is of utmost necessity in a particular model tumor system.

Protective effect of creatine on cardiac mitochondrial respiration inhibition by methylglyoxal

Methylglyoxal is a normal metabolite of glycolytic and other pathways (Talukdar et al. 2009). Our interest to understand the status of creatine–CK system in malignancy stemmed from our studies on the effect of methylglyoxal on mitochondrial respiration of different types of cells, both normal and malignant. We observed that methylglyoxal could arrest mitochondrial electron transport chain at the level of complex I in cancer cells. Surprisingly, methylglyoxal also blocked electron transport of isolated cardiac mitochondria at the level of complex I, whereas it had no effect on mitochondria from several other normal tissues as well as on cardiac tissue slices and intact perfused heart. Kymographic experiments with perfused intact heart had also indicated that methylglyoxal had no effect on several important physiological functions of heart. These results suggested that in cardiac cells there is a protective device to counteract the inhibitory effect of methylglyoxal on cardiac mitochondrial respiration (Ray et al. 1997).

We undertook a study to identify the factor responsible for protection against the inhibitory effect of methylglyoxal on cardiac cellular mitochondria. It had been observed that a post-mitochondrial supernatant of cardiac cells could almost completely protect this inhibition. The endogenous factor that is present in the post-mitochondrial supernatant of cardiac cells responsible for protective effect was finally identified as creatine. Synthetic highly purified creatine also could completely protect cardiac mitochondria from the deleterious effect of methylglyoxal. Interestingly, however, creatine had no protective effect on the inhibition of mitochondrial respiration of rapidly growing, highly dedifferentiated malignant Ehrlich ascites carcinoma (EAC) cells by methylglyoxal. Oxygen consumption by both cardiac and EAC cellular mitochondria were inhibited to the extent of 85% by 2 mM methylglyoxal. But the effect of creatine on these two types of mitochondria was strikingly different. In the presence of 10 mM creatine, the inhibition of cardiac mitochondrial respiration by 2 mM methylglyoxal was reduced to only 15%. In contrast, the same concentration of creatine was totally ineffective in protecting the EAC cellular mitochondrial respiration from the inhibitory effect of methylglyoxal (Roy et al. 2003).

The inhibitory effect of methylglyoxal on mitochondrial complex I of malignant and cardiac cells and the absence of this inhibitory effect on mitochondrial complex I of several normal cells had been investigated further by enzymatic assay of mitochondrial complex I, i.e. the assay of NADH dehydrogenase. Both spectrophotometric and polarographic methods were used. In these assays also, it had been observed that methylglyoxal inactivated NADH dehydrogenase of specifically malignant and cardiac cells

but not of normal liver and skeletal muscle cells. This study was extended with specific amino acid modifying reagents. Whereas, lysine-specific reagents trinitrobenzenesulfonic acid and pyridoxal phosphate inactivated NADH dehydrogenase of specifically malignant and cardiac cells but not of normal liver and skeletal muscle cells (Ghosh et al. 2011).

To sum up the results of these investigations, methylglyoxal inhibits mitochondrial complex I of cardiac and malignant cells, but creatine present in cardiac cells or alternatively also exogenously added creatine could protect against this inhibition of cardiac complex I; whereas malignant cellular mitochondrial complex I could not be protected by creatine. Moreover, enzymatic assays showed that methylglyoxal could inactivate NADH dehydrogenase of cardiac as well as malignant cells but not of the normal cells studied. To conclude, complex I of electron transport chain in malignant cells has some essential similarity with that of cardiac cells in regard to inhibition of electron transport by methylglyoxal and lysine-specific reagents, which are different from that of other normal cells. However, creatine protection is observed only with cardiac cell but not with malignant cells; in this respect complex I of cardiac and malignant cells seem to be different.

Creatine content and CK activity and the status of muscle-specific isoforms of CK in sarcoma tissue and normal contralateral muscle of mice and some normal and malignant post-operative human tissues

The differential effect in protecting cardiac and tumor mitochondria from the deleterious effect of methylglyoxal prompted us to investigate the status of creatine content and CK activity in tumor tissues. We observed that creatine content and CK activity are very low in EAC cells. This raised the question whether in malignant cells the creatine–CK system in general is lower as compared to normal cells.

Moreover, as mentioned above, a systematic study on creatine–CK system seems of utmost necessity in a particular model tumor system. To resolve these issues, we have chosen sarcoma tissue and its normal counterpart (skeletal muscle) to study the status of creatine and CK in malignancy. We experimentally induced sarcoma in one hind leg muscle of mice by either injecting 3-methylcholanthrene, a chemical carcinogen or by inoculating sarcoma 180 cells into one hind leg and compared the creatine content and CK activity in contralateral normal leg muscle and corresponding sarcoma tissue of the same animal with the progression of malignancy. We also investigated the status of creatine content and CK activity in some post-operative human tissues. The normal and malignant nature of human tissues and the progression of malignancy in mice muscle were confirmed by histology (Patra et al. 2008).

We found that in this cancer animal model system the creatine content progressively decreased in sarcoma tissue of mice with the progression of malignancy and reached very low levels in the final stage of tumor development. The activity of CK also followed a similar pattern and was almost undetectable at final stage of tumor development. Interestingly, the levels of creatine and CK activity in a few post-operative tissue samples (fibrosarcoma and gastrointestinal tract malignancy) from human patients were also found to be much reduced compared to healthy control tissues (Patra et al. 2008).

We also tested the status of the two muscle-specific isoforms of CK MCK and sMitCK in sarcoma tissue in mice with the progression of malignancy. When immunoblotting experiments were performed with antibodies against MCK and sMitCK it was observed that both of these isoforms decreased as malignancy progressed. Expressions of mRNA of these two isoforms were also severely downregulated. In human sarcoma these two isoforms were undetectable also (Patra et al. 2008).

Augmentation of the anticancer effect of methylglyoxal plus ascorbic acid by creatine

As mentioned, methylglyoxal had been found to impose strong anticancer activity. Együd and Szent-Györgyi (1968) and Apple and Greenberg (1968) showed long ago remarkable antiproliferative and curative effects of methylglyoxal in cancer-bearing animals. In vitro and in vivo studies with animals and in vitro studies with a wide variety of human post-operative tissue samples we observed that methylglyoxal acted specifically against malignant cells and ascorbic acid significantly augmented this anticancer effect. As already reported, creatine also shows an anticancer effect, so we tested in vivo whether creatine could augment the anticancer effect of methylglyoxal plus ascorbic acid by measuring cancer cell growth inhibition and increase in life span of the animals that had been inoculated with malignant (EAC) cells. It was observed that the anticancer effect of methylglyoxal plus ascorbic acid was significantly augmented by creatine in our in vivo model for cancer. Nearly, 80% of the animals treated with methylglyoxal plus ascorbic acid plus creatine were completely cured and devoid of any malignant cells.

With this background the present study extends our previous work on the curative effect on tumor-bearing mice by methylglyoxal plus ascorbic acid and its augmentation by creatine. Moreover, we also present the status of CK, specially its two isoforms MCK and sMitCK in sarcoma tissue during treatment by these compounds.

Materials and methods

Chemicals, antibody, enzyme and enzyme assay kit

Creatine, nitrocellulose membrane (0.45 μM pore size), anti-rabbit IgG (whole molecule) peroxidase conjugated, anti-mouse IgG (whole molecule) peroxidase conjugated were obtained from Sigma Chemical Co., St. Louis, MO, USA. Anti-α tubulin and luminol reagent were obtained from Santa Cruz Biotechnology, Inc. Santa Cruz, California, USA. M-MLVRT, Taq polymerase, dNTP, random hexamer, Trizol reagent were from Invitrogen, Carlsbad, California, USA. CK assay kit was obtained from Bayer Diagnostics India, Baroda, India. Other chemicals were of analytical grade and obtained from local manufacturers.

Development and treatment of Ehrlich ascites carcinoma and sarcoma tissue in mice

Ehrlich ascites carcinoma

Each mouse was inoculated with 10^6 EAC cells in the peritoneal cavity. The day of inoculation was considered as day 0. Treatment started from day 2 and continued up to day 9.

Sarcoma tissue

Each mouse was inoculated with sarcoma 180 cells (2×10^6) in one hind leg (day 0). Treatment started from day 7 and continued up to day 17.

For both EAC and sarcoma, one group of mice remained untreated and other two groups of mice were treated with methylglyoxal (20) plus ascorbic acid (50) or methylglyoxal (20) plus ascorbic acid (50) plus creatine (150). The amount of each compound indicated in the parenthesis is mg/kg body weight per day. Administration of methylglyoxal was intravenous and aqueous solution of ascorbic acid and creatine feed orally. Each group consists of six mice and received single dose/day. Each set of experiments were repeated four times.

Preparations of total tissue homogenate, cell free extract and mitochondria

Tissue homogenate and chilled native cell free extract

One gram of skeletal muscle from normal mice or sarcoma tissue was taken in 6 ml of pre-chilled 25 mM sodium phosphate buffer, pH 7.4, and homogenized in an Omni GLH homogenizer for a period of 2×1 min with 1 min interval in between. This is considered as 'total homogenate'. Creatine was estimated from this total homogenate. For determination of CK activity in mice muscle and sarcoma tissue, the $650 \times g$ (10 min) supernatant (designated as 'cell free extract') of the above-mentioned total homogenate was used (see below).

Isolation of mitochondria

Mice skeletal muscle or sarcoma tissue was collected and washed in the buffer containing 250 mM sucrose, 1 mM EDTA, and 0.1% BSA and 10 mM Tris, pH finally adjusted to 7.4 by dilute HCl. After finely mincing the tissue, it was homogenized in a Potter-Elvehjem homogenizer with 12 up and down strokes and centrifuged at $650 \times g$ for 10 min. The supernatant was collected and centrifuged at $14{,}000 \times g$ for 10 min. After rejecting the supernatant, the pellet was suspended in the above-mentioned buffer and washed twice by centrifuging at $14{,}000 \times g$ for 10 min. The pellet was suspended in minimum volume of the buffer. An aliquot of the mitochondrial fraction was sonicated for 4×15 s (1 min interval between the pulses) by keeping the fraction on ice. The sonicated 'mitochondrial fraction' was used for assay and immunoblot of mitochondrial isoform sMitCK of CK. Mitochondrial purity was checked by succinate dehydrogenase and glucose-6-phosphate dehydrogenase assay.

Estimation of creatine and assay of CK

Creatine

To 1 ml of total homogenate freshly prepared in the cold, 1 ml of ice-cold 0.6 N perchloric acid was added and immediately centrifuged. After rejecting the pellet, the supernatant was neutralized to pH 7.4 by saturated K_2CO_3 solution. Creatine was estimated in the neutralized supernatant by α-naphthol-diacetyl (Oser 1965).

CK

CK was assayed in the above-mentioned chilled native cell free extract in a coupled enzyme assay system by monitoring the formation of NADPH at 340 nm as per the instructions of manufacturer of the assay kit. The reaction mixture contained, in a total volume of 1 ml, 25 μmol of tris/HCl buffer, pH 7.2, 2.5 μmol of magnesium acetate, 5 μmol of *N*-acetyl-L-cysteine, 0.5 μmol of ADP, 1.25 μmol of AMP, 0.5 μmol of NADP, 5 μmol of D-glucose, 2.5 μmol of diadenosine pentaphosphate, 0.5 μmol of EDTA, 7.5 μmol of phosphocreatine, 8.5 units of hexokinase and 5 units of glucose-6-phosphate dehydrogenase. After 2 min of incubation at 30°C, appropriately diluted aliquots of normal muscle or sarcoma tissue homogenate was added and the change in absorbance was noted from the end of first minute to the end of fifth minute.

Protein was estimated by the method of Lowry et al. as outlined by Layne (1957) with BSA as a standard.

Western blotting

Total tissue homogenate or sonicated mitochondria were separated by SDS-PAGE (7.5%) and transferred to a nitrocellulose membrane. The membrane was blocked for 2 h at room temperature with 5% skimmed milk powder in 50 mM sodium phosphate buffer, pH 7.4 containing 0.9% NaCl (PBS). After washing with PBS containing 2% Tween-20 (PBS-T), the membrane was incubated with diluted primary antibody in PBS overnight at 4°C. After washing again with PBS-T, the membrane was subsequently incubated with secondary antibody in PBS for 1 h at room temperature. The immuno-reactive bands were visualized using Luminol reagent. The primary antibody dilutions used for immunoblot were as follows, 1:5,000 (for MCK), 1:1,000 for sMitCK and α-tubulin. Secondary antibody dilutions were, 1:20,000 anti-rabbit peroxidase-conjugated IgG for MCK, 1:1,000 peroxidase-conjugated anti-mouse IgG for α-tubulin, 1:10,000 peroxidase-conjugated anti-rabbit IgG for sMitCK. Primary polyclonal antibodies against human CK isoforms were produced in rabbits and characterized, as described by Schlattner et al. (2002).

RNA isolation and RT-PCR

Total cellular RNA was prepared from muscle using Trizol regent according to manufacturer's instruction. Single strand cDNA was made from 1 μg of total RNA by using M-MLV reverse transcriptase and random hexamer primer. The cDNA sequence was amplified with specific primer set by PCR using a gene amplification system (Thermocycler, Applied Biosystem 2720). The PCR products were run on 1.5% agarose gel and were visualized by ethidium bromide staining. Internal control for mRNA expression was 18s rRNA. The primers are as follows:

For MCK (Patra et al. 2008)
Forward: 5′-TTC CTT GTG TGG GTG AAC GA-3′
Reverse: 5′-TTT TCC AGC TTC TTC TCC ATC-3′
For sMitCK (Patra et al. 2008)
Forward: 5′-AGG CAG AAG GTA TCT GCT GAT-3′
Reverse: 5′-CCA TGC CCA CAG TCT TAA TGA-3′
For 18s rRNA (Bera et al. 2008)
Forward: 5′-CAC GGC CGG TAC AGT GAA AC-3′
Reverse: 5′-CCC GTC GGC ATG TAT TAG CT-3′

Results

Creatine contents and CK activities in different stages of tumor development in mouse muscle

The creatine content and the activity of CK were measured and compared with normal muscle and sarcoma tissue during the progression of malignancy. We developed sarcoma in the hind leg of mice by injecting 3 methylcholanthrene, a chemical carcinogen. Creatine content was determined in both sarcoma tissue and contralateral normal muscle of the same animal at different stages of tumor development. It had been observed that creatine content and CK activity gradually decreased in the muscles with the progression of malignancy (Fig. 2). In the final stage of in vivo tumor development, creatine contents of sarcoma tissue were almost 90% lower than that of the normal

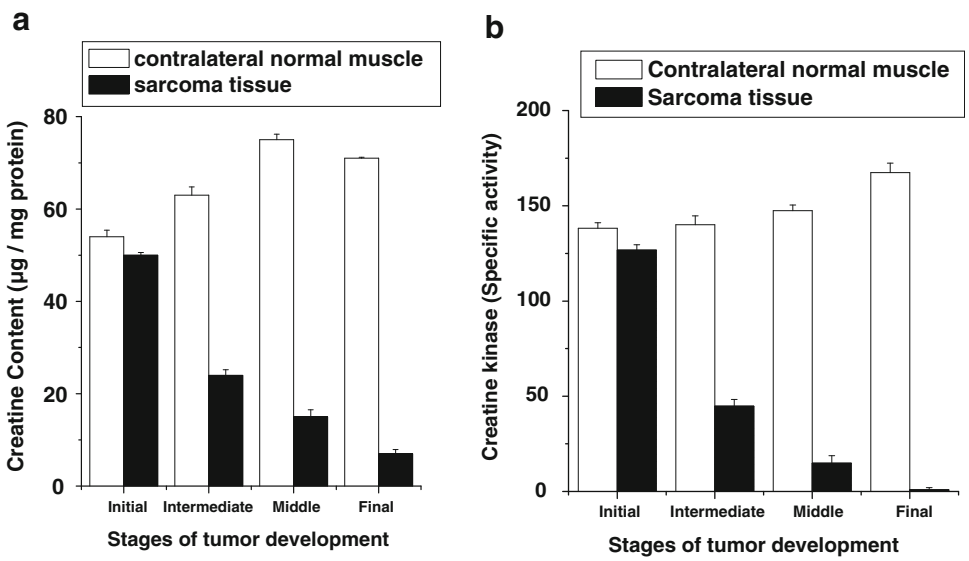

Fig. 2 Creatine content (**a**) and CK activity (**b**) of normal muscle and sarcoma tissue of mice. Creatine content (**a**) and CK activity (**b**) of different stages of tumor development (initial, intermediate, middle and final). In both **a** and **b** *open bars* and *closed bars* represent contralateral muscle of tumor-bearing mice and sarcoma tissue, respectively. The detailed methods of tumor development and different stages of tumor are described in Patra et al. (2008)

Table 1 Treatment of cancer bearing mice (EAC inoculated)

Treatment	Number of EAC cells ($\times 10^6$)					
	2 days	4 days	8 days	10 days	12 days	17 days
None	13 ± 1.1	50 ± 1.4	430 ± 17.3	620 ± 23.8	990 ± 42.7	Died
MG + AA	Do	18 ± 1.3	42 ± 1.9	47 ± 2.2	25 ± 2.0	1.3 ± 1.0
MG + AA + Cr	Do	11.6 ± 0.9	13 ± 1.1	7 ± 0.8	0.1 ± 0.02	No detectable cells

Number of viable cells were counted in a hemocytometer using trypan blue and rechecked by MTT assay. Other details are described in "Materials and methods"

MG methylglyoxal, *AA* ascorbic acid, *Cr* creatine

contralateral muscle of the same animal. The activity of CK also decreased progressively with progression of tumor and that the activity was almost non-detectable in the final stage of dedifferentiation (Patra et al. 2008).

Creatine as an adjuvant in cancer therapy

We investigated the relative regressive effect of methylglyoxal plus ascorbic acid and methylglyoxal plus ascorbic acid plus creatine in mice inoculated with EAC cells. Each mouse was inoculated with 10^6 EAC cells. The number of cells present in the intraperitoneal cavity of mice was counted after 9 days of treatment. It had been observed that in consonance with the absence of any visible tumor in the intraperitoneal cavity no EAC cells could be detected in the mice that were treated with methylglyoxal plus ascorbic acid plus creatine (Table 1). The results presented in Fig. 3 show that the significant curative effect of methylglyoxal plus ascorbic acid was even further augmented by creatine supplementation in EAC cell inoculated mice.

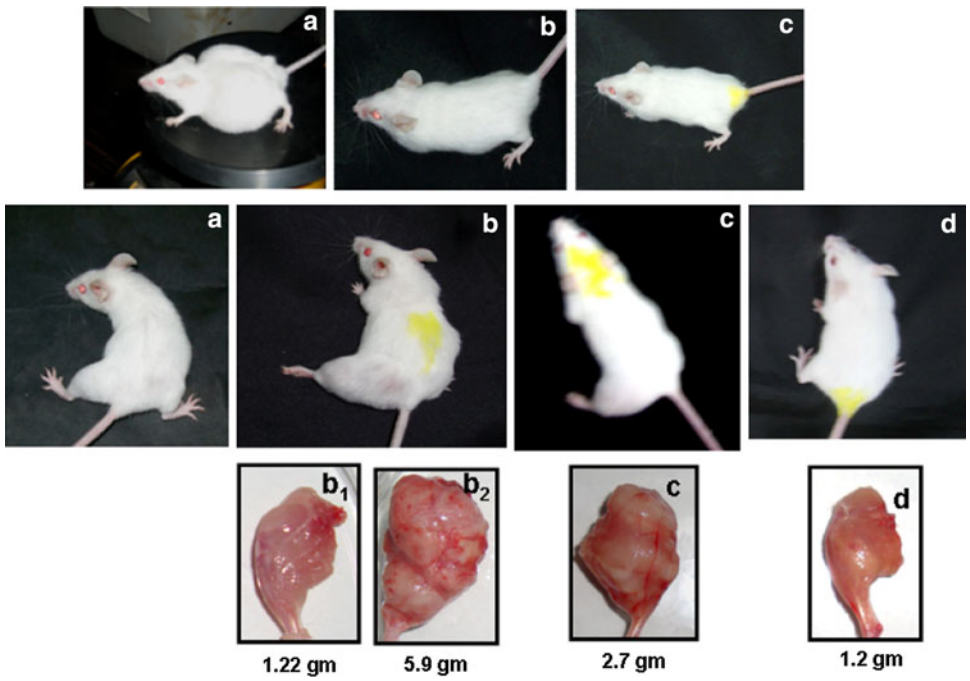

Fig. 3 Treatment of Ehrlich ascites carcinoma or sarcoma 180 cell-inoculated mice with different combinations of methylglyoxal, ascorbic acid and creatine. In the *uppermost panel*, each mouse was inoculated with (10^6) Ehrlich ascites carcinoma cells. Treatment started on day 2 and continued up to day 9: **a** untreated mouse, **b** treated with methylglyoxal plus ascorbic acid, **c** treated with methylglyoxal plus ascorbic acid plus creatine. The *middle panel* shows mice inoculated with sarcoma 180 cells (2×10^6) and the day of inoculation was considered as day 0. Treatment started from day 7 and continued up to day 17. The mice were sacrificed on day 18 and the tumors examined. **a** Untreated animal on day 7, **b** untreated animal (day 18); **c** treated with methylglyoxal plus ascorbic acid (day 18); **d** treated with methylglyoxal plus ascorbic acid plus creatine (day 18). In the *lowest panel* b_1 and b_2 represent, respectively the contralateral muscle and tumor tissue excised from the animal marked **b** in the *middle panel*. In the *lowest panel c* and *d*, respectively represent the tissues excised from the animals marked **c** and **d** in *middle panel*. The respective weights of the excised tissues are indicated. The weight of sarcoma tissue excised from the mouse on day 7 was ≈2.3 g before start of the treatment. Doses and other details of the treatment are described in "Materials and methods"

Table 2 Activity of CK and creatine content in normal muscle and sarcoma tissue and activity of CK and creatinine content in serum of mice treated with methylglyoxal, ascorbic acid and creatine

Treatment	Creatine (μg/mg)		CK (Units/mg protein)		CK (U/ml)	Creatinine (mg/dl)
	Normal muscle (contralateral part)	Sarcoma tissue	Normal muscle (contralateral part)	Sarcoma tissue	Serum	Serum
None (age control mice)	85 ± 12.7		186 ± 19.8		0.2 ± 0.09	1.52 ± 0.6
None	79 ± 1.4	10 ± 5.6	172 ± 5	9.65 ± 2.6	0.7 ± 0.16	1.54 ± 0.4
MG + AA	80.5 ± 4.9	18.5 ± 2.1	162 ± 4.0	30 ± 4.4	0.4 ± 0.16	1.54 ± 0.14
MG + AA + Cr	67.5 ± 0.7	62.0 ± 4.2	164 ± 6	148 ± 18	0.27 ± 0.03	1.6 ± 0.2

The animals were sacrificed on day 18 after receiving 11 days of treatment; creatine and creatinine contents and activity of CK were measured. Other details are described in "Materials and methods"

MG methylglyoxal, *AA* ascorbic acid, *Cr* creatine

We also tested whether treatment of methylglyoxal, ascorbic acid plus creatine had an effect on the regression of sarcoma developed in mice muscle by injecting sarcoma 180 cells. It had been observed that similar to the effect on EAC cell, methylglyoxal plus ascorbic acid could significantly inhibit the growth of tumor mass of the sarcoma 180 cell-induced tumors in vivo. Amazingly, when creatine was added in combination with methylglyoxal plus ascorbic acid the tumor mass reduced to the size of contralateral normal leg muscle indicating total regression of tumor (Fig. 3).

Activity of CK and creatine content in normal muscle and sarcoma tissue and activity of CK and creatinine content in serum of mice treated with methylglyoxal, ascorbic acid and creatine

As shown in Table 2, CK activity and creatine content of muscle of normal mice without tumor inoculation and contralateral control muscle of tumor-inoculated mice are similar. But, both CK activity and creatine content of sarcoma tissue are significantly lower than that of either muscle of normal mice or contralateral muscle of tumor-inoculated mice. When tumor bearing mice was treated with methylglyoxal plus ascorbic acid the activity of CK in the sarcoma was increased significantly. Moreover, when treated with methylglyoxal plus ascorbic acid plus creatine the activity of CK in the area where the sarcoma was developed was increased further and reached to a value near to normal. The creatine content also followed a similar pattern. Interestingly serum CK activity was found to be significantly higher in tumor inoculated untreated group than that of normal mice. But, the serum levels of CK reached an almost normal level in the treated and cured group. However, the serum creatinine content remained almost unchanged in all the cases (Table 2).

Detection of MCK and sMitCK with the decrease of tumor on treatment with methylglyoxal, ascorbic acid and creatine

The drastic reduction of the activity of CK, especially in sarcoma tissue and significant increase on treatment with methylglyoxal, ascorbic acid and creatine prompted us to investigate the status of the two muscle-specific isoforms, MCK and sMitCK in this sarcoma tissue and contralateral normal mice muscle with the decrease of tumor.

Immunoblot experiments of MCK and sMitCK also showed a similar pattern that had been observed with the activity of CK in sarcoma-bearing mice when treated with methylglyoxal, ascorbic acid plus creatine. Both MCK and sMitCK were almost undetectable in sarcoma tissue (Fig. 4) but both muscle CK isoforms reappeared as highly intense bands upon treatment with methylglyoxal plus ascorbic acid and the intensity of these bands increased even further on treatment with methylglyoxal plus ascorbic acid with creatine supplementation.

mRNA expression of MCK and sMitCK along with the decrease of tumor on treatment with methylglyoxal, ascorbic acid and creatine

As mentioned above, we observed that CK is severely down regulated in sarcoma tissue as compared to that of the normal muscle counterpart. The results presented in Fig. 4 clearly indicate that this downregulation is significantly reversed on treatment with methylglyoxal plus ascorbic acid with creatine supplementation. To distinguish whether this down regulation is at the expression level of mRNA or at the level of protein, we determined the mRNA expression levels of MCK and sMitCK by semi quantitative RT PCR in normal mice muscle and sarcoma tissue of the treated mice. The results presented in Fig. 5 show that

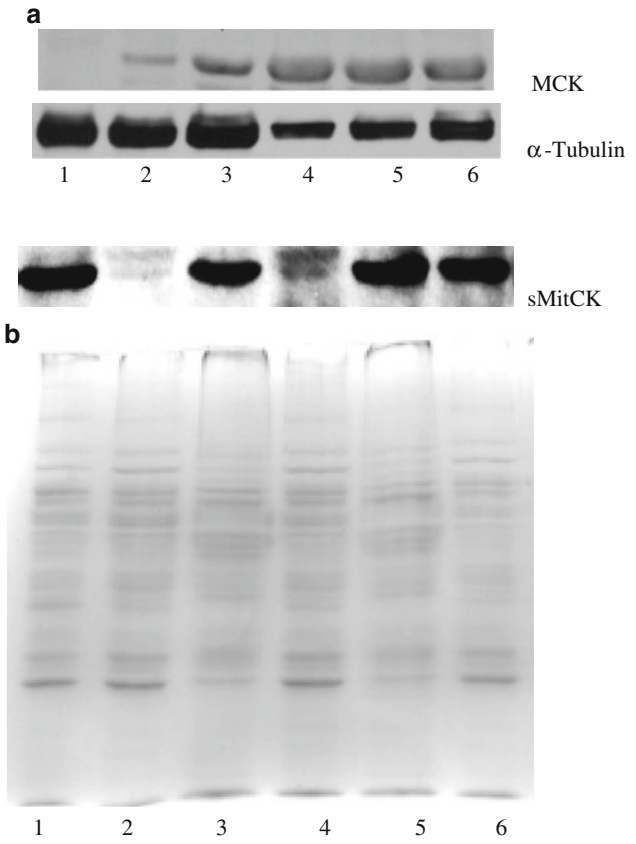

Fig. 4 Immunoblot of MCK (**a**) and sMitCK (**b**) of normal muscle and sarcoma tissue with decrease of tumor on treatment with methylglyoxal, ascorbic acid and creatine. In **a**, *upper panel* MCK of sarcoma tissue of untreated mouse (*lane 1*), mouse treated with methylglyoxal plus ascorbic acid (*lane 2*) and mouse treated with methylglyoxal plus ascorbic acid plus creatine (*lane 3*), *lanes 4, 5, 6* represent the contralateral muscle of the same animal in the same order. *Lower panel* indicates α-tubulin of the corresponding tissues. In **b**, *upper panel* sMitCK of sarcoma tissue of untreated mouse (*lane 2*), mouse treated with methylglyoxal plus ascorbic acid (*lane 4*) and mouse treated with methylglyoxal plus ascorbic acid plus creatine (*lane 6*). *Lanes 1, 3, 5* represent the contralateral muscle of the same animal in the same order. *Lower panel* shows the loading control of mitochondrial protein after SDS-PAGE in the same sequence. Doses and other details of the treatment as well as immunoblotting are described in "Materials and methods"

mRNA expression of both, MCK and sMitCK, are similar in pattern to the activity of CK.

Discussion

The results presented in this paper show that creatine could significantly augment the well known anticancer effects of methylglyoxal plus ascorbic acid. This surprising finding was observed for both EAC cells inoculated in the peritoneal cavity as well as sarcoma tissue in hind leg muscle of mice in vivo. We previously showed that both creatine

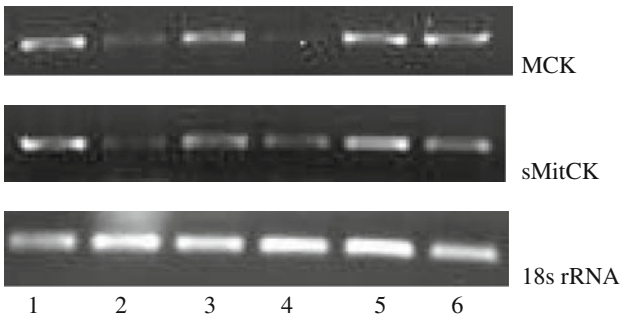

Fig. 5 mRNA expression of MCK and sMitCK of normal muscle and sarcoma tissue with decrease of tumor on treatment with methylglyoxal, ascorbic acid and creatine. In all the three panels, sarcoma tissue of untreated mouse (*lane 2*), mouse treated with methylglyoxal plus ascorbic acid (*lane 4*) and mouse treated with methylglyoxal plus ascorbic acid plus creatine (*lane 6*). *Lanes 1, 3, 5* represent the contralateral muscle of the same animal in the same order. 18s rRNA represents internal control. Doses and other details of the treatment as well as mRNA expression studies are described in "Materials and methods"

content and CK activity of normal muscle progressively decreased upon transformation and progression of malignancy accompanied with progressive loss of the muscle phenotype accompanied by a specific CK-based energy metabolism in the course of de-differentiation (Patra et al. 2008). Immunoblot and mRNA expression experiments unambiguously showed that MCK and sMitCK, the two CK isoforms in adult sarcomeric muscle, progressively decreased with progression of malignancy and were virtually absent in full-grown tumors. This was largely due to reduced gene expression, rather than increased protein degradation (Patra et al. 2008). In the present work, we demonstrate that the curative effect of methylglyoxal plus ascorbic acid on cancer was further augmented with creatine supplementation and that after such combination treatment there was no visible sign of tumor left. It has also been shown that in consonance with the regression of malignancy, the creatine content and the activity of CK as well as expression of the MCK and sMitCK isoforms were restored to almost normal levels.

The mechanism of the surprisingly strong enhancing anticancer effect of creatine to methylglyoxal plus ascorbic acid has not been investigated in the present work. Whether creatine acts on the locus of action of methylglyoxal and further augments its effect or whether it acts via a mechanism independent of the effect of methylglyoxal remains to be established.

There are several reports of the anticancer effect of creatine and cyclocreatine and different explanations such as inhibition of glycolysis (Miller et al. 1993), generation of acidosis (Kristensen et al 1999) had been put forward but none of these studies had investigated the mechanism of action although an apoptosis independent pathway had been suggested (Kornacker et al. 2001).

In this context, we suggest that tumor tissue supplemented with creatine might sequester significant amount of ATP that is necessary for any growth-oriented cells such as malignant cells.

Numerous publications have shown that creatine affects muscle growth and muscle performance. As an ergogenic nutritional supplement creatine reaches the target organs, elevates muscle total creatine and phosphocreatine pools, which leads to an increase in muscle mass and elevates muscle performance(for reviews, Buford et al. 2007; Kerksick et al. 2008). It might be that methylglyoxal destroys the sarcoma tissue and supplementation of creatine restores the normal muscle growth and thus the animal is brought back to the original condition before tumor inoculation.

Besides improving the cellular energy status, creatine displays several energy-independent pleiotropic actions on cells and organelles, and acts as a direct and indirect antioxidant and/or as an anti-apoptotic agent on the so called "mitochondrial permeability transition" (MPT), which is an early event in apoptosis that often leads to swelling of mitochondria and to a release of apoptosis-inducing factors (for review, see Wallimann et al. 2011). Together with other components, MitCK is involved in MPT (Zhivotovsky et al. 2009; Kroemer et al. 2007). Creatine was found not only to stimulate mitochondrial respiration, but similarly to methylglyoxal (Speer et al. 2003), is also involved as an effective mitochondrial protectant and anti-apoptotic compound by directly affecting MPT (Brdiczka et al. 2006). For example, transgenic mice expressing MitCK in their liver acquire, after creatine supplementation, a remarkable tolerance against tumor necrosis factor-induced apoptosis (Hatano et al. 2004). Thus, MitCK plus creatine seem to exert protection to the normal cells against apoptosis (O'Gorman et al. 1997; Brdiczka et al. 2006).

However, our results show that on supplementation of creatine with methylglyoxal plus ascorbic acid, malignant cells are gradually eliminated from the treated animals, which suggest a differential response of normal and malignant cells and points to an intrinsic difference between these two types of cells. The fact that methylglyoxal displays a differential response towards normal and malignant cells had been corroborated by earlier findings. It had been observed that in normal rat liver mitochondria methylglyoxal causes a rapid suppression of MPT (Speer et al. 2003). On the other hand, it had been observed that methylglyoxal induces apoptosis in Jurkat leukemia T cells by activating c-Jun N-terminal kinase (Du et al. 2000).

In this respect, the definite involvement of creatine in MPT in normal versus malignant cells needs further investigation at the molecular level. In addition, it will be necessary to measure comparative ATP, ADP and AMP levels, as well as the expression and activation of AMP-activated protein kinase (AMPK) in treated and untreated cancer cells and in vivo tumors in the cancer model presented in this work, for it would be important to know whether in the cancer model presented here, creatine would act directly on the nucleotide-phosphate level or indirectly by activating AMPK (Neumann et al. 2003; Ceddia and Sweeney 2004), which is directly implied in anti-cancer action (Rocha et al. 2011).

Yet another of the pleiotorpic effects of creatine that may be relevant for the observed anti-cancer action may relate to the action of creatine on the creatine synthesis pathway and the enzymes involved. We have previously shown a prominent up regulation of the expression and enzymatic activity of both AGAT and GAMT of skeletal muscle of mice upon malignant transformation (Bera et al. 2008). The specific activity of AGAT in sarcoma tissue reached almost 50% to that observed in kidney and it had been also observed that the tumor load had no significant effect on the AGAT activity of the kidneys of tumor-bearing mice. Similarly, the specific activity of GAMT in sarcoma tissue was found to be almost equal to that of liver. No change in GAMT activity was observed in liver due to tumor load. Both AGAT and GAMT were also highly detectable in EAC and sarcoma 180 cells and the values were similar to those of sarcoma tissue (Bera et al. 2008).

In addtion, we have shown that the transcript levels of arginase II, ornithine-decarboxylase, *S*-adenosyl-homocysteine hydrolase and methionine-synthase were significantly upregulated in sarcoma and in EAC and sarcoma 180 cells (Bera et al. 2008). Overall, the enzymes related to creatine and arginine/methionine metabolism were found to be significantly upregulated in malignant cells. But, the low levels of CK in the same malignant cells studied seem not to be sufficient for building up an effective creatine/phosphocreatine pool. Instead of supporting creatine biosynthesis, AGAT and GAMT seem to be geared to support cancer cell metabolism in the direction of polyamine and methionine synthesis as both these compounds are in high demand in proliferating cancer cells.

As sarcoma development consequently transformed the requirement of fluctuating energy demand of a normal muscle to a more constant, continuous energy demand of a tumor, the creatine–CK system seems less important or obsolete. Moreover, creatine acts as a feedback inhibitor of AGAT (da Silva et al. 2009), a function less likely to be advantageous for tumor growth. Thus, the high activity of creatine synthesizing enzymes and the low levels of creatine in cancers do not represent a dichotomy but rather are most likely a pathway towards tumorigenesis.

In conclusion, (a) the level of creatine and CK might be used as a marker of malignancy of sarcomeric muscle tissue, (b) high activity of creatine biosynthesizing enzymes and low level of CK may represent a general trend in

cancer favoring pathways towards tumorigenesis, (c) creatine supplementation might be used as an adjuvant in cancer therapy and (d) the downregulation of CK observed in sarcomas needs further detailed investigation. It will be interesting to see whether the unambiguous trend with downregulation of the CK system and at the same time upregulation of the creatine synthesis enzymes holds true also for other cancers growing in non-muscle tissue.

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