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Progressive decrease of phosphocreatine, creatine and creatine kinase in skeletal muscle upon transformation to sarcoma

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In vertebrates, phosphocreatine and ATP are continuously interconverted by the reversible reaction of creatine kinase in accordance with cellular energy needs. Sarcoma tissue and its normal counterpart, creatine-rich skeletal muscle, are good source materials to study the status of creatine and creatine kinase with the progression of malignancy. We experimentally induced sarcoma in mouse leg muscle by injecting either 3-methylcholanthrene or live sarcoma 180 cells into one hind leg. Creatine, phosphocreatine and creatine kinase isoform levels decreased as malignancy progressed and reached very low levels in the final stage of sarcoma development; all these parameters remained unaltered in the unaffected contralateral leg muscle of the same animal. Creatine and creatine kinase levels were also reduced significantly in frank malignant portions of human sarcoma and gastric and colonic adenocarcinoma compared with the distal nonmalignant portions of the same samples. In mice, immunoblotting with antibodies against cytosolic muscle-type creatine kinase and sarcomeric mitochondrial creatine kinase showed that both of these isoforms decreased as malignancy progressed. Expressions of mRNA of muscle-type creatine kinase and sarcomeric mitochondrial creatine kinase were also severely downregulated. In human sarcoma these two isoforms were undetectable also. In human gastric and colonic adenocarcinoma, brain-type creatine kinase was found to be downregulated, whereas ubiquitous mitochondrial creatine kinase was upregulated. These significantly decreased levels of creatine and creatine kinase isoforms in sarcoma suggest that: (a) the genuine muscle phenotype is lost during sarcoma progression, and (b) these parameters may be used as diagnostic marker and prognostic indicator of malignancy in this tissue.

In biological systems, ATP is the universal energy currency. Excitable cells and tissues, such as skeletal and cardiac muscle, brain, photoreceptor cells, spermatozoa and electrocytes all depend on the immediate availability of vast amounts of energy that may be used in a pulsed or fluctuating manner [1,2]. Because the adenylate pool and the ATP : ADP ratio are key regulators influencing many fundamental metabolic

Abbreviations
3MC, 3-methylcholanthrene; BCK, brain-specific creatine kinase; CK, creatine kinase; DAB, 3,3′-diaminobenzedine tetrahydrochoride; MCK, muscle-specific creatine kinase; sMitCK, sarcomeric mitochondrial creatine kinase.

processes, these cells avoid building up a large pool of ATP. Instead, large quantities of 'metabolically inert' phosphagens are stored in these cells or tissues. In vertebrate species, phosphocreatine is the sole phosphagen and ATP is continuously and efficiently replenished from the large pool of phosphocreatine via a reaction catalyzed by creatine kinase (EC 2.7.3.2) [3].

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

In vertebrates, creatine kinase (CK) is present as four different isoforms [1–4] and creatine is synthesized sequentially by a two-step process. The first step takes place in the kidney and/or pancreas by the enzyme L-arginine : glycine amidino transferase (EC 2.1.4.1). The product of this reaction, guanidinoacetic acid, is transported to the liver and converted to creatine by the enzyme *S*-adenosyl-L-metheonine-*N*-guanidinoacetate methyl transferase (EC 2.1.1.2). Creatine is then released from the liver into the blood stream via an unknown mechanism. In cells requiring creatine, a specific Na^+- and Cl^--dependent creatine transporter is responsible for the uptake of creatine across the plasma membrane. This transporter is predominant in heart, skeletal and smooth muscle, as well as in brain and some other organs [2].

In rapidly growing cells, such as malignant cells, demand for ATP is extremely high and more or less continuous, in contrast to excitable cells which have a fluctuating energy requirement. Because creatine metabolism is intimately connected with ATP requirements, its role in malignant cells is of prime importance. However, there are conflicting results on the role of the CK/creatine system in tumor growth and malignancy [5–20]. Here we analyze whether changes in the phosphocreatine/creatine system are related to the progression of sarcoma malignancy, a question that has not been studied systematically to date.

During our previous studies on the effect of methylglyoxal and creatine on mitochondrial respiration in Ehrlich ascites carcinoma and cardiac cells, we observed that both creatine content and CK activity were very low in these rapidly growing, highly dedifferentiated malignant cells [5]. Literature data on creatine content and CK expression in malignant cells and tumor-bearing animals, however, give a somewhat ambiguous picture. There are reports, especially in the older literature, of increases in creatine content in malignant tissues and in tumor-bearing animals [6–8]. Some comparative studies on the activities of different CK isoforms in some normal and malignant cells also indicate mixed results. Some studies report upregulation of some form(s) of CK in malignant cells [9–14]. By contrast, there have been reports of decreased levels of creatine and an increased choline/creatine ratio, and a decrease in the activity of CK and some of its isoforms in several different forms of cancer [15–20].

Skeletal muscle tissue contains high levels of phosphocreatine, creatine and CK, and this tissue can be transformed into malignant sarcoma tissue by injecting the chemical carcinogen, 3-methylcholanthrene (3MC). That this procedure yields malignant sarcomas in the muscles of one leg contralateral to normal leg muscle allows us to obtain tumor and normal control material from the very same tissue in the same animal. We developed sarcoma using such a procedure, and also by injecting existing sarcoma 180 cells that had been previously maintained in the peritoneal cavity of mice. Analyses of phosphocreatine, creatine and CK and its isoforms in sarcoma tissue with tissue of progressing malignancy revealed significant changes relative to untreated, unaffected muscle. We also measured the creatine content, as well as the CK activity in a limited number of post-operative biopsy samples from human cancers. These studies indicate a general downregulation of the CK system in malignant tissues.

Results

Comparative creatine and phosphocreatine content and CK activity in normal muscle and sarcoma tissue of mice

As mentioned above, we had previously observed that both creatine content and CK activity are very low in Ehrlich ascites carcinoma cells, a rapidly growing, highly dedifferentiated malignant cell [5]. We had also made similar findings with sarcoma 180 cells (unpublished observation). In this study, we measured and compared the creatine and phosphocreatine content, as well as the CK activity, in normal muscle and sarcoma tissue during the progression of malignancy. The sarcoma was developed in the hind leg of mice by injecting 3MC, a chemical carcinogen, or by injecting into the hind leg existing sarcoma 180 cells, which had been previously maintained in the peritoneal cavity of mice. Creatine content was measured in both sarcoma tissue and contralateral normal muscle of the same animal at different stages of tumor development.

If we compare the creatine and phosphocreatine content and CK activity present in normal, healthy muscle at different stages of malignancy of the contralateral leg, we observe a gradual increase in these parameters in normal muscle from initial (postnatal age 10–12 weeks) to final stage (postnatal age 24–26 weeks). This increase appears to be due to the

increased creatine and phosphocreatine content and CK activity in healthy differentiated muscle with increasing age of the experimental animals. Approximately, 14 and 6 weeks are needed to develop a full-grown tumor by 3MC and sarcoma 180 cells, respectively. We measured creatine and phosphocreatine content and CK activity at 0, 7, 10 and 14 weeks, in the sarcoma group and an age-matched control group. We observed increases in creatine and phosphocreatine content and CK activity with the progression of age in the healthy contralateral hind-limb muscles (data not shown).

However, as shown in Table 1, both creatine and phosphocreatine progressively decreased in the muscles as both carcinogen- and cell-induced malignancy progressed. In the final stage of tumor development, both the creatine and phosphocreatine content of sarcoma tissue was almost 90% lower than in the normal contralateral muscle of the same animal. Table 2 shows that CK activity also decreased progressively with tumor development and that was almost non-detectable in the final stage. It appears that the biochemical changes matched the histological changes (Fig. 1).

In this study, we used two different methods of tumor induction, administering carcinogen, 3MC, and inoculating with tumor cells. The 3MC compound might produce some secondary effects in the animal in addition to tumor induction. During multiplication inoculated malignant cells could completely displace the normal tissue of the host. However, we observed a reduction in creatine, phosphocreatine and CK in both carcinogen- and cell-induced malignant cells. This suggests that this reduction is directly linked to malignancy and is not due to a carcinogen-induced secondary effect or to the displacement of normal muscle cells by sarcoma 180 cells that contain low creatine, phosphocreatine and CK.

Creatine content and CK activity in postoperative human normal and malignant tissue samples

We also investigated the status of creatine and CK in a few postoperative tissue samples from human patients, the results of which are presented in Table 3. Similar to the results presented in Tables 1 and 2, we observed that the creatine content and CK activity were much reduced in both human fibrosarcoma and gastrointestinal tract malignancy, compared with healthy control tissues, and that CK activity was almost non-detectable in fibrosarcoma (Table 3). Figure 2 shows the histological sections of normal colon and adenocarcinoma of colon tissue from a patient.

Immunoblot experiments

In vertebrates, four different CK isoforms are expressed in a tissue-specific manner [1–4]. There are two cytosolic CK isoforms. Whereby 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 such as photoreceptor cells and lens of eyes, spermatozoa, intestinal epithelia etc. In addition, there are two mitochondrial (MitCK) isoforms. Sarcomeric sMitCK is co-expressed with MCK in striated skeletal and heart muscles and ubiquitous uMitCK is present in smooth muscle, brain, neuronal and other non-muscle

Table 1. Creatine and phosphocreatine content in normal muscle (NM) and sarcoma tissue (ST) of mice induced by 3MC and sarcoma 180 cells. ND, not detected.

	Creatine content ($\mu g \cdot mg^{-1}$ protein)			Phosphocreatine content ($\mu g \cdot mg^{-1}$ protein)		
Stages of tumor development[a]	NM[b]	ST	Reduction (%)[c]	NM[b]	ST	Reduction (%)[c]
3MC						
Initial	54 ± 1.4	54 ± 0.6	0.6	112 ± 4.0	96 ± 3.0	14
Intermediate	63 ± 1.8	24 ± 1.2	26	120 ± 3.2	41 ± 2.1	66
Middle	75 ± 1.2	15 ± 1.5	80	119 ± 1.2	33 ± 6.7	72
Final	71 ± 0.2	7 ± 0.9	90	125 ± 1.5	12 ± 5.7	91
Sarcoma 180 cells						
Initial	53.6 ± 1.2	52.4 ± 1.0	2.2	102.4 ± 2.6	82.3 ± 18	19.6
Middle	74.4 ± 2.3	13.3 ± 2.1	82.1	ND	ND	
Final	83.8 ± 2.7	9.6 ± 0.4	88.5	114.5 ± 2.9	8.49 ± 0.9	92.6

[a] Stages of tumor development described in Experimental procedures. [b] Creatine content was measured in contralateral normal muscle of the same animal at different stage of tumor development. [c] Percentage of reduction in ST compared with NM at the same stage of tumor development.

Table 2. CK activity in normal muscle (NM) and sarcoma tissue (ST) of mice induced by 3MC and sarcoma 180 cells.

Stages of tumor development	Creatine kinase (Specific activity)		
	NM[a]	ST	Reduction (%)[b]
3MC			
Initial	138.2 ± 2.9	126.7 ± 2.9	8.3
Intermediate	140 ± 4.7	44.8 ± 3.5	32.0
Middle	147.5 ± 2.9	14.9 ± 3.9	89.9
Final	167.5 ± 4.9	0.9 ± 1.1	99.4
Sarcoma 180 cells			
Initial	125.1 ± 3.0	120.9 ± 3.7	3.3
Middle	142.6 ± 1.2	10.9 ± 1.9	92.3
Final	164.5 ± 3.4	0.6 ± 0.8	99.0

[a] CK content was measured in contralateral normal muscle of the same animal at different stages of tumor development. [b] Percentage of reduction in ST compared with NM at the same stage of tumor development.

tissues and is co-expressed with BCK [4]. The drastic reduction in CK activity, especially in sarcoma tissue prompted us to investigate the status of these four isoforms in this sarcoma tissue with the progression of malignancy.

Detection of MCK and sMitCK in normal muscle and sarcoma tissue with progression of malignancy

Immunoblotting experiments show that MCK decreased progressively with the development of malignancy in mouse muscle tissue (Fig. 3A). In the final stage of sarcoma development, MCK was almost undetectable (Fig. 3A,d). Figure 3B shows the results of a similar experiment with sMitCK used to probe mitochondrial preparations of normal muscle and sarcoma tissue. Because in samples of the final phase of tumor development the CK isoenzymes were below the detection limit of 3,3′-diaminobenzedine tetrahydrochoride (DAB)-stained immunoblots, we also used the more sensitive chemiluminescence (Luminol) method. However, even with the chemiluminescence method, MCK and sMitCK remained undetectable in late-stage sarcoma samples. In healthy control tissue, both MCK and sMitCK isoenzymes were unambiguously detect-

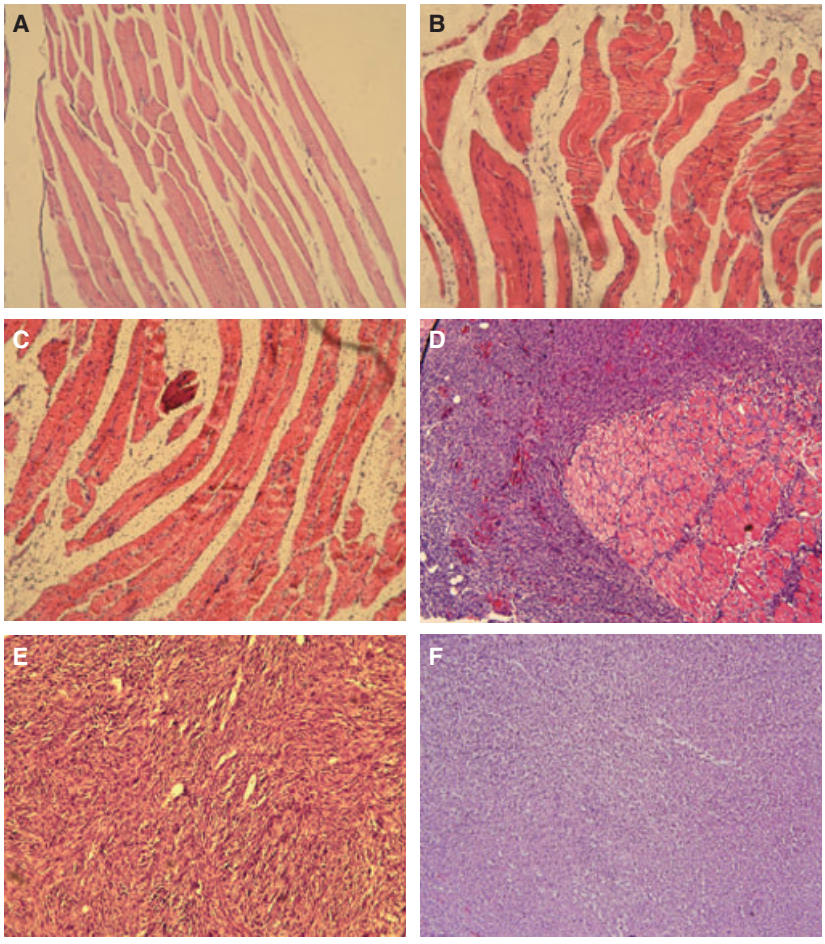

Fig. 1. Histological examination of normal mouse muscle (A) and different stages of sarcoma induced by 3MC. (B) Initial stage, (C–E) different intermediate stages with progression of malignancy, (F) full-grown tumor. The stains used were eosin and hematoxylin.

Table 3. Creatine content and creatine kinase activity in post-operative human normal (N) and malignant tissue (T) samples.

Organ/type of malignancy (no. of samples)	Creatine kinase (sp. activity)			Creatine content ($\mu g \cdot mg^{-1}$ protein)		
	N	T	Reduction (%)[a]	N	T	Reduction (%)[a]
Colon/adenocarcinoma (4)	1.6 ± 0.9	0.6 ± 0.2	62.5	11.5 ± 6.2	4.7 ± 0.7	59.1
Colon/melanoma (1)	3.56	0.31	91.3	4.8	1.2	75.0
Stomach/adenocarcinoma (3)	1.2 ± 0.4	0.5 ± 0.3	58.3	5.2 ± 2.0	2.7 ± 2.5	48.0
Skeletal muscle/fibrosarcoma (3)	42.4 ± 3.3	0.02 ± 0.002	99.9	34	7.7	77.3

[a] Values indicate the percentage of reduction of creatine/CK in normal and malignant samples as compared to the normal counterpart.

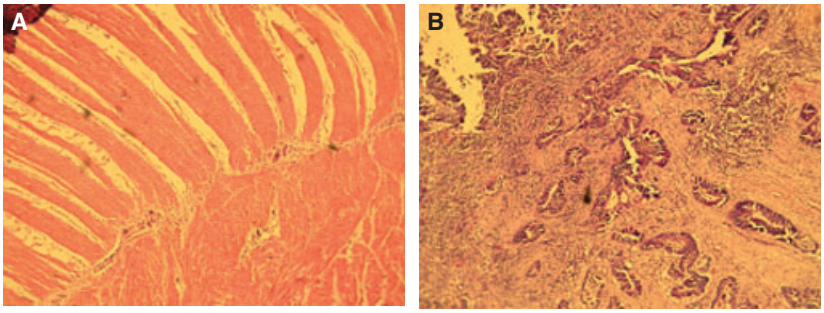

Fig. 2. Histological examination of normal colon (A) and adenocarcinoma (B) of colon from a postoperative human specimen. The stains used were eosin and hematoxylin.

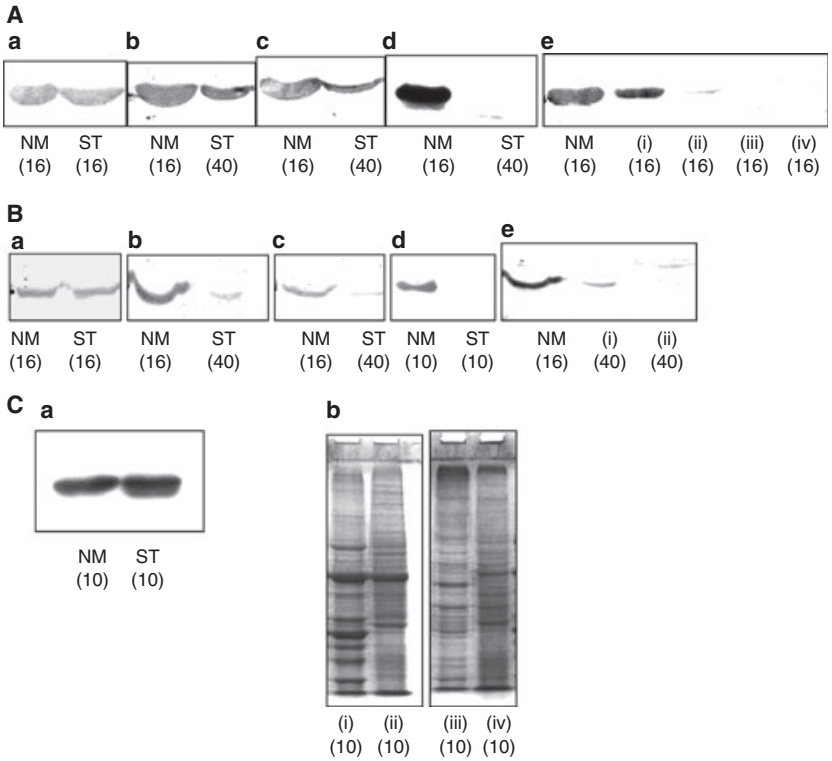

Fig. 3. Immunoblot of MCK (A) and sMitCK (B) of normal mouse muscle (NM) and sarcoma tissue (ST) at different stages of tumor development. In both (A) and (B), (a–d) represent sarcoma tissue developed by 3MC; (e) represents tumor induced by sarcoma 180 cells (in e: i–iv/i–ii different progressive stages of sarcoma tissue). (Ca) α-Tubulin as a control for protein transfer and (Cb) for protein loading. In (C-b), (i) and (ii) represent normal and tumor muscle homogenate respectively; (iii) and (iv) represent normal and tumor mitochondria respectively. Protein bands were developed with Coomassie Brilliant Blue stain. In both (A) and (B), DAB was used for visualization of immunoreactive bands in (a), (b), (c) and (e), whereas luminol reagent was used for (d). The values in the parentheses at the bottom of each panel indicate μg of protein applied.

able by the antibodies at the appropriate subunit M_r of ~ 42–45 kDa.

When a similar immunoblot experiment was performed with postoperative human normal muscle and sarcoma samples, again a very prominent MCK (Fig. 4A,a) and sMitCK (Fig. 4A,b) band with an apparent M_r of ~ 45 kDa was observed in healthy control tissue, whereas these bands were totally absent

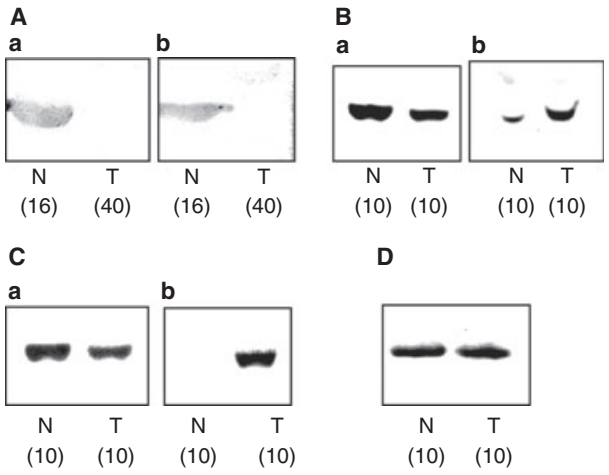

Fig. 4. Immunoblot of CK isoforms of different post-operative human tissues. (A) Immunoblot of MCK (a) and sMitCK (b) of normal muscle (N) and fibrosarcoma (T) tissue. (B) Immunoblot of BCK (a) and uMitCK (b) of normal stomach (N) and gastric adenocarcinoma (T). (C) Immunoblot of BCK (a) and uMitCK (b) of normal colon (N) and colonic adenocarcinoma (T). (D) Immunoblot of cytochrome *c* oxidase I of normal colon (N) and colonic adenocarcinoma (T). In (A) bands were visualized by DAB. In (B–D) Luminol reagent was used for visualization of immunoreactive bands. Values in the parentheses represent μg protein applied.

in sarcoma samples. Note that because of the low abundance of MCK and sMitCK isoenzymes in sarcoma, a much higher amount of protein had to be applied compared with normal muscle.

Detection of BCK and uMitCK isoforms in human gastric and colorectal adenocarcinoma tissues

BCK protein expression in both gastric (Fig. 4Ba) and colonic (Fig. 4Ca) malignant tissue homogenates was less than in the normal counterpart. However, with the uMitCK isoform the situation is reversed in these tissues (Fig. 4Bb,Cb). Immunoblot experiments with antibodies against uMitCK indicate overexpression of this isoform in gastric and colonic adenocarcinoma. Similar overexpression of uMitCK had been observed in different tumor cell lines [21,22].

We had used α-tubulin (Fig. 3C) and cytochrome *c* oxidase I (Fig. 4D) antibody as the control for protein transfer in the case of cytosolic and mitochondrial preparations respectively and Coomassie Brilliant Blue staining (Fig. 3Cb) as the protein loading control. It had been found that cytochrome *c* oxidase I protein expression in human samples remained the same in both normal and malignant colon and stomach mitochondria (Fig. 4D). However, in mouse samples there are reports of the upregulation of cytochrome *c* oxidase I expression in a CK-deficient mouse model [23]. We found similar results in mouse sarcoma tissue mitochondrial preparation (data not shown).

mRNA expression of different CK isoforms in normal muscle and sarcoma tissue

The results presented above clearly indicate that CK is severely downregulated in sarcoma tissue compared with the normal muscle counterpart. To distinguish whether this downregulation is at the level of mRNA expression or at the level of protein turnover, we quantified mRNA expression levels using both semi-quantitative RT-PCR and quantitative real-time PCR of different CK isoforms in normal mouse muscle and sarcoma tissue induced by 3MC. The results presented in Fig. 5 show that mRNA expression of both MCK and sMitCK are severely downregulated. Real-time RT-PCR also shows that the tumor mRNA expressions of MCK and sMitCK were severely downregulated by 100× and 70× respectively with respect to normal muscle mRNA (Fig. 6).

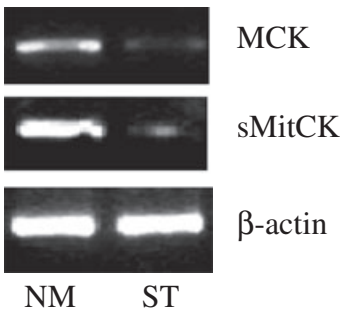

Fig. 5. Expression of mRNA of MCK and sMitCK isoforms of creatine kinase and β-actin in normal muscle (NM) and 3MC induced sarcoma tissue (ST) of mouse.

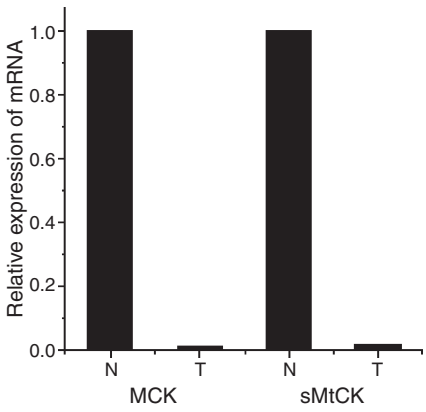

Fig. 6. mRNA expression of MCK and sMitCK by real-time RT-PCR. N and T represent normal and full grown sarcoma induced by 3MC respectively.

Discussion

In this study, we investigated the status of creatine and CK mainly in sarcoma tissue with the progression of malignancy compared with the normal muscle counterpart in the contralateral leg of the same animal. The results clearly indicate that in both carcinogen-induced and sarcoma 180 cell-induced sarcoma, creatine, phosphocreatine and CK gradually decreased in the sarcoma tissue as the tumor mass increased; in the apparently unaffected contralateral normal muscle of the same animal these parameters remained unchanged. The levels of creatine and CK were also significantly reduced in the frank malignant portion of postoperative tissues compared with distal tissues from the same samples.

Immunoblot and mRNA expression experiments of MCK and sMitCK, the two CK isoforms in adult muscle, unambiguously showed that these isoforms progressively decreased as malignancy progressed and were virtually absent in full-grown tumors. This was largely because of reduced gene expression, not increased protein degradation. Together with the decreased creatine content in malignant sarcoma, this is indicative of a progressive loss of the muscle phenotype with its specific CK-based energy metabolism over the course of de-differentiation into malignant sarcoma tissue, which also loses the contractile properties of the original muscle tissue.

Furthermore, our results indicate that the BCK isoform of adenocarcinoma of two organs, stomach and colon, is also downregulated. Here, a special mention is needed about the BCK isoform in relation to malignancy. In many tumors, although not all, BCK activity had been found to be considerably higher than in the tissue of origin [9–14]. Its elevated activity had been suggested as a marker for several malignancies such as small-cell lung carcinoma [12] and neuroblastoma [13]. However, estrogen and other hormones and growth factors had been found to stimulate BCK activity in target cells [24]. Increased CK activity had been also observed in regenerating rat liver [25]. In this study, we observed a downregulation of BCK in colonic and gastric adenocarcinoma. However, it will be necessary to investigate in more detail the status of BCK in brain and other neurological malignancies. Overall, these results suggest that BCK cannot be used as a global marker for malignancy. Moreover, factors not be directly linked with malignancy may influence BCK expression.

It had been observed that uMitCK is overexpressed in different tumor cells [21,22]. It had also been reported that uMitCK inhibits the mitochondrial permeability transition pore and thereby inhibits apoptosis in tumor cells [22]. We found similar overexpression of uMitCK in human colonic and gastric adenocarcinoma. Because uMitCK by its presence between the mitochondrial inner and outer membranes can regulate the mitochondrial permeability transition pore complex [26], overexpression of uMitCK and subsequent inhibition of the mitochondrial permeability transition pore may be a mechanism to counteract apoptosis.

As mentioned above, previous publications from different laboratories had shown some ambiguous results concerning the levels of creatine and CK in malignant cells and in tumor-bearing animals. In some recent publications, the ratio of choline to creatine has been measured in several types of brain tumors [17–19]. It was observed that this ratio is significantly higher in malignant cells, suggesting that the absolute value of creatine may be lower in these cells than in normal cells. This is supported by our observation that phosphocreatine and creatine are very low in sarcoma tissue and significantly lower in all human carcinomas studied. Because creatine is synthesized in tandem in kidney, pancreas and liver and is transported to different target organs [1,2] normal levels of phosphocreatine, creatine and CK are unaffected in normal skeletal muscle of the same tumor-bearing animal, as observed in this study. This raises the possibility that the creatine transporter that allows entry of creatine into the muscle may be downregulated in sarcoma malignancy.

However, it has been reported that Ehrlich ascites tumor cells could transport significant amounts of creatine and cyclocreatine when incubated in presence of these compounds [27]. Moreover, this cell type can phosphorylate large amounts of these phosphagens 'under favorable conditions'. However, similar to the findings of Roy *et al.* [5], Ehrlich ascites tumor cells contain very low levels of CK compared with skeletal and cardiac muscles [27]. It may be that, because of the intrinsic low level of CK in at least some malignant cells, these cells are unable to retain their intracellular creatine pool in the form of phosphocreatine. The simultaneous decrease in phosphocreatine, creatine and CK in sarcoma tissue suggests that creatine availability and CK levels may be regulated in a coordinate manner. Moreover, the creatine transporter may be a target of the carcinogen for the development of malignancy. However, a systematic study of creatine-synthesizing enzymes, CK and the creatine transporter in relation to malignancy is of urgent necessity.

Various studies [28,29] have shown that in many tumor cell types the level of p53 protein is decreased due to mutation of its gene. Also, p53 protein generated from p53 tumor suppressor gene has a binding affinity to a novel recognition sequence in the proximal promoter of the MCK gene, and this binding activates the transcription of MCK. Hence, it can be assumed that reduction/alteration in p53 protein in many tumor types is one reason for the lower level of MCK production by those cells; this may also be the case in our model where sarcoma-bearing muscle shows a much lower level of MCK than its normal counterpart.

There are reports of anticancer effects for creatine and its analogs such as cyclocreatine [30,31]. The question naturally arises whether phosphocreatine generation by the CK reaction has a general role in the anticancer effect. In rapidly growing cells, such as malignant cells, the demand for ATP is significantly higher and adenylate energy charge has a profound influence on cellular metabolism. The creatine/CK system is highly expressed in excitable cells that have a high and fluctuating energy demand, such as skeletal and cardiac muscles, nerve, retina or sperm [1–3]. By contrast, cancer cells may resemble the liver more, an organ that is virtually devoid of creatine/CK. Both require energy in a much more constant way, without the fluctuations as seen in muscle or nerve cells. In undifferentiated sarcoma tissue, which is growth oriented, the contractile and excitable properties of differentiated normal muscle tissues are expected to be lost, and thus possibly also the requirement for a functional creatine/CK system. These conclusions are fully in line with the drastic reduction in creatine, phosphocreatine and CK in sarcoma tissue as observed in this study. It should be mentioned, however, that this hypothesis mainly applies to MCK/sMitCK-expressing sarcomas that we analyzed here in detail. BCK/uMitCK-expressing tumors often have at least one isoform strongly expressed, mostly the mitochondrial one (see above).

A final question arises in this context, namely whether muscle tissues by virtue of their high creatine content exert a negative control on cellular proliferation? Despite the presence of a very high amount skeletal muscle tissue, the incidence of sarcoma is very rare. In the absence of any clear evidence we can only speculate that the high level of creatine may participate in such a negative control of uncontrolled cell proliferation. The progressive decrease in creatine and CK in sarcoma tissue with the spread of malignancy suggests that this hypothesis should be tested as a diagnostic marker and prognostic indicator for sarcoma, at least.

Experimental procedures

Chemicals, antibody, enzyme and enzyme assay kit

Creatine, phosphocreatine, 3MC, DAB, nitrocellulose membrane (0.45 μm pore size), primers, anti-(rabbit IgG) (whole molecule) peroxidase conjugated and anti-(mouse IgG) (whole molecule) peroxidase conjugated were obtained from Sigma Chemical Co. (St Louis, MO, USA), anti-(cytochrome *c* oxidase I), anti-(α-tubulin) and anti-(goat IgG) peroxidase conjugated and luminol reagent were obtained from Santa Cruz Biotech. (Santa Cruz, CA, USA). M-MLVRT, *Taq* DNA polymerase, dNTP, random hexamer and Trizol reagent were from Invitrogen (Carlsbad, CA, USA). Power SYBR green master mix was obtained from Applied Biosystems (Foster City, CA, USA). The CK assay kit was obtained from Bayer Diagnostics India (Baroda, India). Other chemicals were of analytical grade and obtained from local manufacturers.

Development of sarcoma tissue in the hind leg of mice

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

3MC was dissolved in hot olive oil and 0.1 mL containing $\sim$ 0.2 mg of the carcinogen (10 mg·kg^{-1} body weight) was injected into one hind leg of Swiss albino mice. This procedure was repeated twice more with a 1-week interval between the injections. Sarcoma tissue was also developed in the hind leg of a mouse by injecting sarcoma 180 cells (2×10^6 cells) that had previously been maintained in the intraperitoneal cavity of Swiss albino mice. The third or fourth day after third (final) injection was considered to be the initial phase of tumor development. When tumor weight reached $\sim$ 7–9 g or half this weight, it was considered to be the final or middle phase of tumor growth, respectively. The time needed to reach full-grown tumor by 3MC and sarcoma 180 cells was 14 and 6 weeks, respectively. Malignancy was confirmed by histological examination (Fig. 1). It appears from Fig. 1 that the muscle-specific appearance of the tissue was gradually lost during tumor development. Importantly, the number of cells and mitotic figures increased with progressive stages. Figure 1D shows a significant change with the appearance of hemorrhagic tissue indicating the onset of malignancy; this was considered an intermediate stage of tumor growth.

Postoperative normal and malignant human tissue

Postoperative human tissue samples were collected in cold normal saline immediately after surgery and brought to the

laboratory on ice. The experiments were started within half an hour of surgery. Frankly malignant tissues were compared with the distal part of the operated tissues where there was no evidence of malignancy. These distal tissue materials were considered as normal (Fig. 2).

Preparation of total tissue homogenate, cell-free extract and mitochondria

Tissue homogenate and cell-free extract

One gram of skeletal muscle from normal mice or sarcoma tissue, or postoperative human samples, both normal and malignant was taken in 6 mL of prechilled 25 mM $NaCl/P_i$ 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 to be 'total homogenate'. Creatine was estimated from this total homogenate. To determine phosphocreatine and CK activity in mouse muscle and sarcoma tissue, and human tissues, the 650 ***g*** (10 min) supernatant (designated '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 was finally adjusted to 7.4 using dilute HCl. For postoperative human tissue, the buffer used was 70 mM sucrose, 210 mM mannitol, 1 mM EGTA, 10 mM Hepes, 0.1% BSA, pH 7.4. After finely mincing the tissue, it was homogenized in a Potter-Elvehjm homogenizer with 12 up-and-down strokes and centrifuged at 650 ***g*** (1500 ***g*** for human tissue) for 10 min. The supernatant was collected and centrifuged at 14 000 ***g*** for 10 min (8000 ***g*** for 15 min for human tissue). After rejecting the supernatant, the pellet was suspended in the above-mentioned buffer and washed twice by centrifuging at 14 000 ***g*** for 10 min (8000 ***g*** for 15 min for human tissue). 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 pulse) by keeping the fraction on ice. The sonicated 'mitochondrial fraction' was used for assay and immunoblot of mitochondrial isoforms (sMitCK and uMitCK) of CK. Mitochondrial purity was checked by succinate dehydrogenase and glucose-6-phosphate dehydrogenase assay.

Estimation of creatine, phosphocreatine and assay of CK

Creatine

To 1 mL of freshly prepared (in the cold) total homogenate, 1 mL of ice-cold 0.6 M 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 [32].

Phosphocreatine

Phosphocreatine was immediately estimated in the above-mentioned chilled cell-free extract. An appropriately diluted aliquot from this cell-free extract was incubated at 65 °C for 1 min. Then 0.2 mL of 0.4 M HCl was added and incubated for 9 min at 65 °C after which 0.2 mL of 0.4 M NaOH was added. In the extract treated in this way, phosphocreatine is completely hydrolysed to creatine. After cooling, total creatine was then determined photometrically. The phosphocreatine content was calculated by subtracting the measured creatine content of a similar homogenate without prior acid hydrolysis and neutralization.

Creatine kinase

Creatine kinase was usually estimated in the above-mentioned chilled cell-free extract without the addition of Triton, or alternatively, the CK content was estimated by extracting either normal muscle or sarcoma tissue after homogenization in 6 vol of a buffer containing 25 mM Tris, 30 mM dithiotreitol, 0.3 M sucrose and 1% Triton X-100, the pH was finally adjusted to 8.0 using diluted HCl. The extraction was carried out for 1 h by keeping the homogenate on ice. The CK activity, if measured immediately, was found to be similar in both of these fresh and chilled preparations.

Creatine kinase was assayed in a coupled enzyme assay by monitoring the formation of NADPH at 340 nm as per the instructions of the assay kit manufacturer. The reaction mixture contained, in a total volume of 1 mL, 25 μmol of Tris/HCl buffer, pH 7.2, 2.5 μmol magnesium acetate, 5 μmol *N*-acetyl-L-cysteine, 0.5 μmol ADP, 1.25 μmol AMP, 0.5 μmol NADP, 5 μmol D-glucose, 2.5 μmol diadenosine pentaphosphate, 0.5 μmol EDTA, 7.5 μmol phosphocreatine, 8.5 units hexokinase and 5 units glucose-6-phosphate dehydrogenase. After 2 min 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 estimation was carried out with BSA as a standard by the method of Lowry *et al.* as outlined by Layne [33].

Western blotting

Total tissue homogenate or sonicated mitochondria were separated in 7.5% polyacrylamide/SDS gels 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 (NaCl/P_i). After washing with NaCl/P_i containing 2% Tween-20 (NaCl/P_i-T), the membrane was incubated with diluted primary antibody in NaCl/P_i overnight at 4 °C. After washing again with NaCl/P_i-T, the membrane was subsequently incubated with secondary antibody in NaCl/P_i for 1 h at room temperature. The immunoeactive bands were visualized using either Luminol reagent or DAB. The primary antibody dilutions used for immunoblot were as follows, 1 : 5000 (for MCK) or 1 : 1000 for BCK, sMitCK, uMitCK, cytochrome *c* oxidase I and α-tubulin. Secondary antibody dilutions were, 1 : 20 000 peroxidase-conjugated anti-(rabbit IgG) for MCK, 1 : 1000 peroxidase-conjugated anti-(mouse IgG) for BCK and α-tubulin, 1 : 10 000 peroxidase-conjugated anti-(rabbit IgG) for sMitCK and uMitCK and 1 : 1000 peroxidase-conjugated anti-(goat IgG) for cytochrome *c* oxidase I. Primary polyclonal antibodies against human creatine kinase isoforms were produced in rabbits and characterized, as described by Schlattner *et al.* [34] and a monoclonal antibody against human BCK, prepared according to the method of Sistermans *et al.* [35].

RNA isolation and RT-PCR

Total cellular RNA was prepared from muscle using Trizol reagent according to manufacturer's instructions. 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. β-Actin was used as an internal control for mRNA expression. The primers and respective product size are as follows. For MCK (434 bp) [36]: forward, 5'-TTCCTTGTG TGGGTGAACGA-3'; reverse, 5'-TTTTCCAGCTTCTTCT CCATC-3'. For sMitCK (226 bp) [37]: forward, 5'-AGGCA GAAGGTATCTGCTGAT-3'; reverse, 5'-CCATGCCCAC AGTCTTAATGA-3'. For β-actin (514 bp) [38]: forward, 5'-TGTGATGGTGGGAATGGGTCAG-3'; reverse, 5'-TT TGATGTCACGCACGATTTCC-3'.

Real-time RT-PCR

Real-time RT-PCR was carried out using the above cDNA preparation with Power SYBR green master mixture kit (Applied Biosystems) and an Applied Biosystems 7500 Real Time PCR System according to the manufacturer's recommended protocol. The parameter Ct was defined as the fractional cycle number at which the fluorescence generated by passing a fixed threshold above baseline. The Ct values were obtained from real-time PCR machine after the run for different sets of sample. From these Ct values we calculated the relative mRNA expression by the formula $2^{-\triangle\triangle Ct}$ as described previously [39]. The fold change of the tumor mRNA was normalized to β-actin and relative to normal mRNA were calculated using by the $2^{-\triangle\triangle Ct}$ formula. Primers for real time PCR were designed from GENE FISHER software as follows. For MCK (214 bp): forward -5'-TCAACCACGAGAACCTC A-3'; reverse, 5'-TCCGTCATGCTCTTCAGA-3'. For sMitCK (217 bp): forward, 5'-CAAACTGGAGTGGACA AC-3'; reverse, 5'-GAGAGGACAACACATAGC-3'. For β-actin (348 bp): forward, 5'-TGGAATCCTGTGGCATC CATGAAAC-3'; reverse, 5'-TAAAACGCAGCTCAGTAA CAGTCCG-3'.

Statistical analysis

The number of animals in each group was four, and five such groups were maintained for the measurement of creatine, phosphocreatine and CK in mice. Results are presented as mean ± SD. Student's *t*-test was used to compare the data of normal and tumor samples. $P < 0.005$ was considered statistically significant.

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