Int. J. Cancer: **94,** 513–519 (2001)

Publication of the International Union Against Cancer

HODGKIN DISEASE-DERIVED CELL LINES EXPRESSING UBIQUITOUS MITOCHONDRIAL CREATINE KINASE SHOW GROWTH INHIBITION BY CYCLOCREATINE TREATMENT INDEPENDENT OF APOPTOSIS

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Ubiquitous mitochondrial creatine kinase (uMtCK), a key enzyme in energy metabolism, was identified by differential display PCR to be specifically overexpressed in L1236, the first cell line of definite Hodgkin origin. RT-PCR confirmed overexpression of uMtCK in the L1236 cell line and the absence of cytosolic B-CK, which is co-expressed with MtCK physiologically. Cyclocreatine (cCr), whose phosphorylated form is a very poor substrate for CK, inhibited proliferation of the L1236 cell line nearly entirely. This inhibition by cCr was partially reversed by competition with creatine, which by itself had no effect on proliferation of the L1236 cell line. Although these results support a role of CK activity in the inhibitory action of cCr, it remains open whether the cCr effect is due to its inhibition of CK-linked energy metabolism or if alternative mechanisms have to be considered. Because the anti-proliferative effect of cCr was not due to induction of apoptosis, in contrast to most other anticancer agents, treatment with the creatine analogue cCr may represent an advantageous therapeutic approach for cells resistant to programmed cell death.

Key words: *Hodgkin disease; gene expression; creatinekinase; apoptosis*

In Hodgkin disease (HD), the telltale Hodgkin and Reed-Sternberg (H-RS) cells are surrounded by reactive tissue components. The scarcity of the malignant cells has hampered their genetic analysis. Only recently, single cell PCR analysis could show a clonal B-cell origin of the H-RS cells.[1] Sequence analysis demonstrated that these cells lost their immunoglobulin (Ig) expression due to crippling mutations acquired in the germinal centre. Normal B-cells unable to express Ig are prevented from antigenic selection and are eliminated by apoptosis. The transforming event in HD allowing the cells to escape from programmed cell death is not known. One possible candidate is Epstein-Barr virus (EBV), which is found in 50% of HD cases and has been shown to upregulate the antiapoptotic gene bcl-2 in infected cells.[2] In addition, the complex chromosomal aberrations seen in HD[3] may cause changes in gene expression resulting in resistance to apoptosis. Because of the considerable karyotypic variation from case to case, different molecular mechanisms may be involved in malignant transformation in HD and, in support of this, no HD specific genetic aberration could be identified so far.

One of the mechanisms possibly involved in malignant transformation is overexpression of creatine kinase (CK). The enzyme reversibly catalyzes the transfer of a phosphate group from ATP to creatine (Cr) and occurs as different isoforms. The cytosolic brain-type isoform (B-CK) is coexpressed with the ubiquitous mitochondrial isoform (uMtCK) in many cells and tissues with a high energy demand like brain, placenta, kidney, testis, sperm or endothelial cells.[4] Overexpression of CK has been demonstrated in a wide variety of solid tumors[5–8] and tumor cell lines.[9] Elevated levels of CK have been detected in the sera of patients with advanced-stage cancer and are characteristic for a poor prognosis.[6,8,10] CK catalyzes the reversible transphosphorylation between ATP and creatine: creatine + ATP ↔ phosphocreatine + ADP. The enzyme is thought to have 2 main functions in energy metabolism.[4] It buffers the cellular ATP pool by maintaining high cytosolic concentrations of phosphocreatine (PCr), which can be used in times of high cellular energy demand for the regeneration of ATP and it maintains an energy shuttle between subcellular sites of energy supply (oxidative phosphorylation, glycolysis) and sites of energy demand using the easily diffusable Cr/PCr. For example, MtCK, located in the mitochondrial intermembrane space, preferentially catalyzes the phosphorylation of Cr to PCr, which then diffuses into the cytosol where it is used in the reverse reaction by cytosolic CK to restore ATP for different, often closely associated ATPases.[4,11] In addition to these enzymatic functions, MtCK has been implicated in the regulation of the Ca^{2+}-induced mitochondrial permeability transition pore (PTP).[12] PTP is involved in triggering the execution of apoptosis by the release of pro-apoptotic factors into the cytosol.[13] With isolated mitochondria, the presence of active octameric MtCK was able to prevent or at least delay the opening of PTP.

Cyclocreatine (1-carboxymethyl-2-iminoimidazolidine, cCr) is a synthetic analogue of creatine (Cr) and is converted at the same rate as creatine to the phosphorylated form, but the product phosphocyclocreatine (PcCr) is 160-fold less efficient than phosphocreatine at sustaining ATP production through CK.[14] cCr treatment of CK-positive tumor cells has been shown to slow cell proliferation *in vitro* and *in vivo*,[15] to reduce colony formation in soft agar[15] and to inhibit tumor cell motility.[16] Substantial evidence suggests that the presence of CK activity is necessary for the inhibitory action of cCr.[15,17] This suggests that trapping of high-energy phosphates in the PcCr pool may lead to an impairment of cellular energy metabolism. cCr treatment does not necessarily alter overall NTP concentrations, however,[16,18] and Cr was shown

Abbreviations: B-CK, brain cytosolic isoenzyme of CK; BM, bone marrow; cCr, cyclocreatine; CK, creatine kinase; Cr, creatine; DD, differential display; EBV, Epstein-Barr-virus; HD, Hodgkin disease; H-RS cell, Hodgkin-Reed-Sternberg cell; Ig, immunoglobulin; LCL, lymphoblastoid cell line; M-CK, muscle cytosolic isoenzyme of CK; PBMNC, peripheral blood mononuclear cells; PcCr, phosphocyclocreatine; PCr, phosphocreatine; sMtCK, sarcomeric mitochondrial isoenzyme of CK; uMtCK, ubiquitous mitochondrial isoenzyme of CK.

Grant sponsor: Dr. Mildred Scheel Stiftung fuer Krebsforschung, Germany; Grant sponsor: Swiss National Science Foundation; Grant number: 3100-50824.97; Grant sponsor: Swiss Cancer League.

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Received 7 December 2000; Revised 4 May, 27 June 2001; Accepted 29 June 2001

in mice to have similar inhibitory effects as cCr in solid, transplanted tumors.[18,19]

We recently established the cell line L1236 from the peripheral blood of a patient with mixed cellularity HD.[20] The L1236 cell line is identical, regarding the rearranged Ig gene sequence, with the Reed-Sternberg cells in the bone marrow of the same patient.[1] This cell line shows the phenotypic features of H-RS cells, *i.e.*, an activated phenotype with expression of CD30 and has a biallelic Ig heavy chain and a monoallelic Ig kappa light chain gene rearrangement. EBV transformed B-cells (lymphoblastoid cell lines, LCL) also have rearranged Ig-genes and an activated phenotype with CD 30 expression similar to H-RS cells, but their karyotype is normal and they are not tumorigenic after s.c. inoculation into nude mice.[21]

In our study, we describe the use of differential display (DD) RT-PCR[22] to compare mRNA expression patterns of 3 Hodgkin disease derived cell lines (L1236, L540, L428) and 2 human EBV-immortalized B-cell lines L1309 and L1311. We report the identification and functional characterization of overexpression of MtCK in HD derived cell lines.

MATERIAL AND METHODS

Cells

HD derived cell lines have been reviewed by Drexler.[23] Cell lines KMH2, HDLM2, BJAB,[24] Jurkat[25] and DU-145[26] were purchased from the DSMZ, Braunschweig, Germany. Cell lines BL60[27] and 87/4[28] were obtained from J.B. Jones, S. Poppema, Groningen, G. Lenoir, Lyon and K. Thalmeier, Munich, respectively. All other cell lines were established at the Department I of Internal Medicine, University of Cologne. Tissue origin of the cell lines is summarized in Table I. Cell lines were grown at 37°C with 5% CO_2 in RPMI 1640 (Gibco-BRL, Eggenstein, Germany) supplemented with 2 mM glutamine and 10% fetal calf serum. Primary cells were obtained from healthy volunteers. Peripheral blood mononuclear cells (PBMNC) were isolated from buffy coat cells by Ficoll-Hypaque density centrifugation. B-cell enrichment and depletion of CD3+ cells from PBMNC was achieved by using magnetic cell sorting on mini-Macs columns and anti-CD19 and anti-CD3 beads (Miltenyi, Bergisch Gladbach, Germany) as recommended by the manufacturer. RNA of germinal centre B-cells, cultured dendritic cells and cardiac muscle was kindly provided by U. Klein, Cologne, S. Titzer, Cologne and J. Zehelein, Heidelberg, respectively.

Differential display RT-PCR

Total RNA was isolated using the RNeasy® Mini Total RNA Kit (Qiagen, Hilden, Germany). Cells (1×10^7) were processed per spin column. RNA (1 μg) was reverse transcribed with SuperScript™ RT (Gibco) using either primer (5′-AAGCTTTTT-TTTTTTC-3′), (AAGCT11G) or (AAGCT11A). The reaction was performed at 42°C for 45 min and then terminated by heating to 75°C for 5 min. One-tenth of the resulting cDNA was amplified with recombinant Taq-Polymerase (Gibco) in the presence of (α-32P) dATP with the same poly-T primer and 1 of 7 arbitrary primers: 5′-TTCGAACTAACGG-3′, 5′-TTCGAATTGCCAG-3′, 5′-TTCGAACAGGTAG-3′, 5′-TTCGAACAGGTAG-3′, 5′-TT-CGAAGGCGTTA-3′, 5′-TTCGAATCCAGTG-3′, 5′-TTCGA-ACGCATAC-3′. PCR consisted of 40 cycles of denaturation at 94°C for 30 sec, annealing at 40°C for 40 sec and extension at 72°C for 30 sec. In this setting, 105 different cDNA populations (5 cell lines × 21 [3 × 7] primer combinations) were generated. cDNAs were separated on an 8% sequencing gel, in the presence of 6 M urea, transferred to blotting paper and exposed to Kodak X-OmatAR film over night. cDNA fragments present in HD-derived cell lines were cut out from the gel, eluted by boiling in H_2O for 30 min, precipitated by ethanol using glycogen as a carrier and subsequently reamplified by PCR using the same primer combination. The selected cDNAs were subcloned into pCR2.1 vector by TA-cloning® (Invitrogen, San Diego, CA) and transformed into *E.coli* INV-α-F′ cells (Invitrogen). Clones were sequenced using the DyeDeoxyTerminater cycle sequencing kit (Perkin-Elmer, Weiterstadt, Germany) and an ABI Primus 377 Sequencer (Applied Biosystems, Weiterstadt).

TABLE I – TISSUE ORIGIN OF CELL LINES

Tissue of origin	Cell lines
Classical HD	L1236, L428, L540, HDLM2, KMH2
EBV transformed B-cells	L1309, L1310, L1311, LauI
Burkitt lymphoma	BJAB, BL60
T-cell leukemia	Jurkat
Bone marrow stroma	87/4
Prostate carcinoma	DU-145

Detection of creatine kinase

RT-PCR was performed as for differential display RT-PCR, but primers used were random hexamers (Gibco) for reverse transcription, 5′-CTACTCCAGGATCCCGTAGC-3′ and 5′-TCGGAG-GTCTGGGTACTCAG-3′ for uMtCK, 5′-TCTCCAACAGCCA-CAACGCA-3′ and 5′-GAGGTCGGTCTTGTGCTCA-3′ for B-CK, 5′-GGAGACTCCATCTGGCTTC-3′ and 5′-TCCGT-CATGCTCTTCAGAG-3′ for the muscle-type cytosolic isoform (M-CK), 5′-ATGCTTCTCTGCTGTTTGC-3′ and 5′-AAGGT-CAGCAAACACCTCA-3′ for the sarcomeric mitochondrial CK (sMtCK) and 5′-GTGAAGGTCGGAGTCAACG-3′ and 5′-GGT-GAAGACGCCAGTGGACTC-3′ for GAPDH. For human β-Actin, the RT-PCR Control Amplimer Set (Clontech, Palo Alto, CA) was used. Annealing temperatures were 58°C for uMtCK and B-CK, 53°C for GAPDH and 61°C for M-CK, sMtCK and β-actin. MgCl2 concentration was always 1.5 mM. After 35 PCR cycles products were separated on a 2% agarose gel and visualized with UV-light after ethidiumbromide staining.

Western blotting

Rabbit muscle M-CK (Roche Molecular Biochemicals, Mannheim, Germany) and highly purified, recombinant human B-CK, sMtCK and uMtCK[29] were separated together with extract of the L1236 cell line by standard 12% SDS-PAGE and electrotransferred by semidry blotting (Trans Blot SD, Bio-Rad, CA) onto nitrocellulose (0.45 μm pore size; Schleicher & Schüll, Dassel, Germany) according to the manufacturer's instructions. Loading of the blots was verified by a reversible staining with Ponceau S (0.2% in 0.3% TCA; Serva, Heidelberg, Germany). Membranes were blocked with 4% fat-free milk powder in TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl), incubated for 1 hr with rabbit immune sera against human M-CK, human sMtCK and human uMtCK (1:1,000 dilution in blocking buffer) or affinity-purified chicken IgY against human B-CK (1:500 dilution in blocking buffer), washed 3 times in TBS, incubated 1 hr with peroxidase-coupled secondary antibodies (1:3,000 dilution in blocking buffer), either goat anti-rabbit IgG (Nordic, Lausanne, Switzerland) or rabbit anti-chicken IgY (Jackson ImmunoResearch, West Grove, PA) and finally washed 3 times with TBS. Blots were developed with enhanced chemiluminescence substrate (NEN, Zaventem, Belgium) and signals recorded with a digital imager (Kodak Image Station; Kodak, Rochester, NY).

pH-Stat method for creatine kinase activity

CK activity was determined with a pH stat (Radiometer Copenhagen, Villeurbanne, France), measuring proton consumption in the CK reverse reaction with 4 mM ADP and 10 mM PCr at 25°C.[30]

Cell proliferation assay

Cells/well (1×10^4) were plated in 100 μl cell culture media with or without cCr or Cr into a 96 well plate. ^{3}H-thymidine (0.5 μCi) was added on day 2 and incorporation into DNA was measured after harvesting onto glass fiber plates 16 hr later.

Apoptosis assay

Cells (1×10^5) were plated in 200 μl cell culture media with or without cCr into a 48-well plate. After 3 days binding of fluorescein labeled Annexin V to the cell membrane was quantified by flow cytometry (Apoptosis Detection Kit, R&D, Minneapolis, MN). Ten thousand events were collected on a Becton Dickinson (San Jose, CA) FACScan and Annexin V positive events were quantified using FloJo software. % apoptosis compared to control cells not treated with cCr was calculated according to the following formula:

$$\text{apoptosis (\%)} = \frac{(\%\ \text{apoptosis in cCr treated cells}) - (\%\ \text{apoptosis in control cell})}{100 - (\%\ \text{apoptosis in control cells})}$$

RESULTS

Differential display RT-PCR

We compared gene expression of the Hodgkin-derived cell lines L1236, L428 and L540 with 2 EBV transformed lymphoblastoid B-cell lines (LCL), using 21 primer pairs consisting each of 1 of 3 poly-T oligonucleotides and 1 of 7 random primers. Thirty-six bands corresponding to mRNAs expressed in 1 or more of the HD cell lines, but absent from LCLs, were isolated. All isolated bands were cloned, sequenced and compared to sequences and expressed sequence tags of the DNA Data Bank of Japan, the EMBL Nucleotide Sequence Database, GenBank and Protein DataBank using the BLAST 2.0 server of the National Center of Biotechnology Information (USA). Of the 36 sequences analyzed, 16 matched to expressed sequence tags (EST) and 14 revealed no significant homology to any sequence in the databases, thus representing novel genes. Four sequences harbored DNA of the cloning vector and 2 sequences matched to known genes. The 2 genes identified were tryptophanyl-tRNA synthetase and ubiquitous mitochondrial creatine kinase (uMtCK), the latter 1 detected in the L1236 cell line, but not in L428 and L540. We are currently investigating the expression pattern of the other genes and focus here on the analysis of CK expression and its role as a potential antitumor target.

Expression of creatine kinase isozymes in Hodgkin-derived cells

Expression of MtCK protein in the L1236 cell line was confirmed by Western blotting. A band at the expected size of 43 kD could be demonstrated using an anti-human uMtCK antibody. No B-, M- or sMtCK was detected (Fig. 1). Semiquantitative RT-PCR on a panel of 5 HD derived cell lines, 8 transformed B-cell lines and primary B-cells and 7 other cell populations confirmed the overexpression of uMtCK in the L1236 cell line (Fig. 2*a*). A strong RT-PCR signal for uMtCK was generated in 2 of the 5 HD lines tested (L1236, HDLM2) and a weak band was detected in 1 more HD line (L540). Overall, uMtCK expression was found in 3 of 5 HD derived cell lines but in none of the other 15 cell populations. Because of the weak expression of both uMtCK and B-CK in the RT-PCR assay cell line L540 was omitted from further analysis.

If overexpression of creatine kinase played a role in an energy shuttle between mitochondria and cytosol, a coexpression of uMtCK and cytosolic CK might be expected.[4] We used RT-PCR to analyze B-CK expression in the same panel of 20 cell populations, but only 1 HD line was positive for B-CK (KMH2). Of the other 15 cell populations, 2 LCL (L1310, LauI) and BL60 Burkitt lymphoma cells were weakly positive for B-CK (Fig. 2*a*). No expression of the other 2 CK isoenzymes, cytosolic muscle CK (M-CK) or sarcomeric mitochondrial CK (sMtCK) could be detected in the L1236 cell line by RT-PCR (Fig. 2*b*).

CK enzyme activity was detected in whole cell lysates of the HD lines expressing the enzyme. Using the pH-stat method,[4] we found 0.2 U/mg protein in the L1236 cell line and a lower activity in KMH2 and HDLM2 cells (Fig. 3).

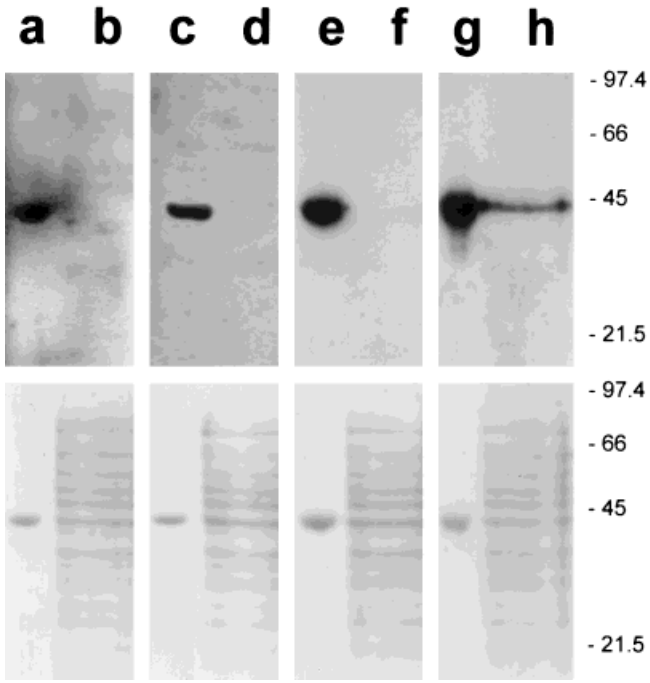

FIGURE 1 – Immunoblots of purified CK isoenzymes and extracts from cell line L1236. Lanes contain (a) 2 μg rabbit M-CK, (c) 2 μg human B-CK, (e) 3 μg human sMtCK, (g) 3 μg human uMtCK, or (b, d, f, h) 20 μg L1236 extract. Blots were immunostained (top panel) using polyclonal antibodies against (a, b) human M-CK; (c, d) human B-CK, (e, f) human sMtCK and (g, h) human uMtCK. Equal protein loading and transfer were verified by reversible Ponceau S staining (bottom panel).

Cyclocreatine treatment of HD cells

The creatine analogue cyclocreatine (cCr) is phosphorylated by creatine kinase to yield the synthetic phosphagen *N*-phosphocyclocreatine (PcCr). Accumulation of PcCr has been shown to inhibit proliferation of a variety of tumor cells.[15] We tested the effects of cCr on the uMtCK-positive HD cell lines, using DU-145 prostate carcinoma cells (CK activity 1.6 U/mg protein[15]) as positive control (Fig. 4). L428, lacking CK activity, served as a negative control. After 3 days of incubation in 12.5 mM cCr, the proliferative capacity of the L1236 cell line was markedly reduced by approximately 60%. KMH2 and DU-145 cell lines also showed a reduced thymidine uptake upon cCr treatment, albeit to a lesser extent. cCr had no significant effect on the CK negative L428 cell line. HDLM2 cells, despite their expression of uMtCK, were resistant to cCr treatment.

Creatine partially reverses the effect of cyclocreatine on Hodgkin-derived cell lines

If the observed growth inhibition were specifically caused by cCr treatment leading to a slow turnover rate of its phosphorylated form PcCr, then incubation with the physiological substrate creatine should be able to reduce the cCr effect. Therefore, the Hodgkin-derived cell lines were incubated with various concentrations of cCr and, at the same time, Cr was added at a concentration of 50 mM (Fig. 5). Indeed, ^{3}H thymidine uptake was partially restored in all cell lines that were sensitive to cCr, although such an effect could not be detected in L428 and HDLM2 cells.

Creatine itself does not promote tumor cell proliferation

As Cr reduced the antiproliferative effect of cCr, we set out to determine if it may promote cell growth when used alone (Fig. 6). Cr at a concentration of 12.5, 25 and 50 mM had no significant

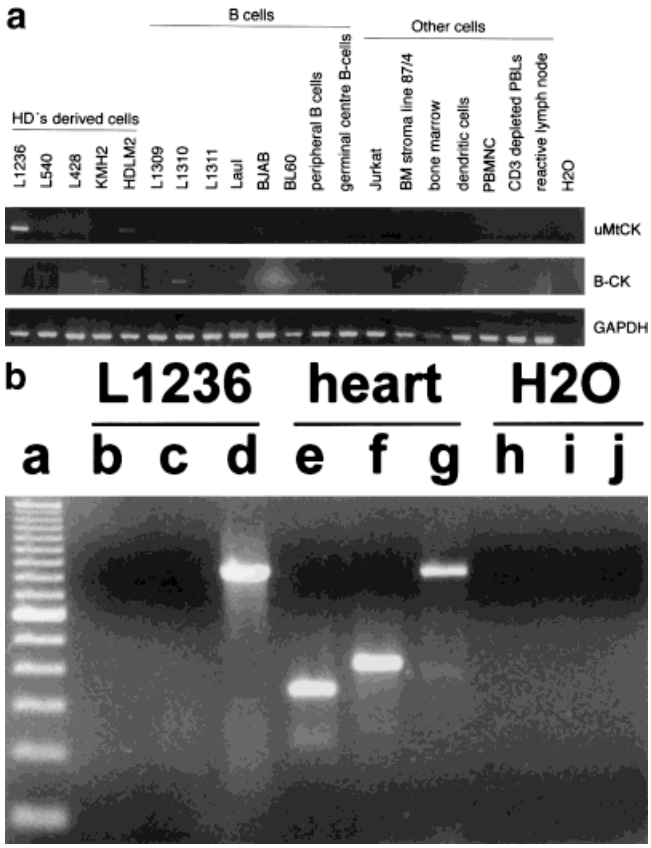

FIGURE 2 – (*a*) Semiquantitative RT-PCR. RT-PCR was performed to detect expression of uMtCK and B-CK on a panel of 5 HD derived cell lines, 8 transformed B-cell lines and primary B-cells and 7 other cell populations. BM and PBMNC stand for bone marrow and peripheral blood mononuclear cells, respectively. (*b*) RT-PCR for SMTCK (*b,e,h*) and M-CK (*c,f,i*) and β-actin (*d,g,j*) in the L1236 cell line (*b,c,d*). cDNA from cardiac muscle (*e,f,g*) served as positive, H_2O (*h,i,j*) as negative control. The expected fragment length is 329 bp for SMTCK, 408 bp for M-CK and 838 for β-actin. To estimate size, a 100 bp ladder was used (*a*).

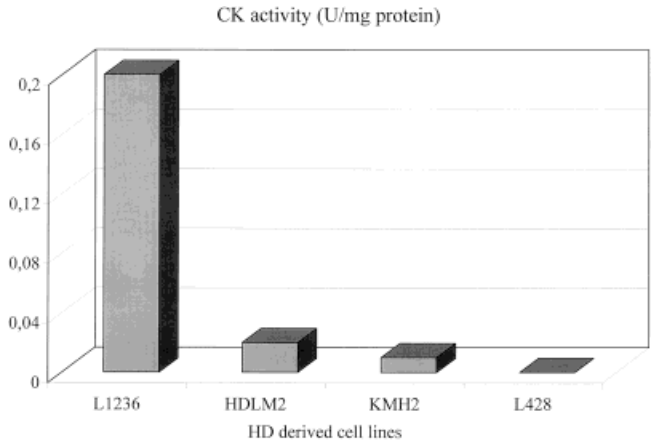

FIGURE 3 – Creatine kinase activity (U/mg protein) in HD derived cell lines was measured with the pH stat method in whole cell lysates. SEM never exceeded ± 10%.

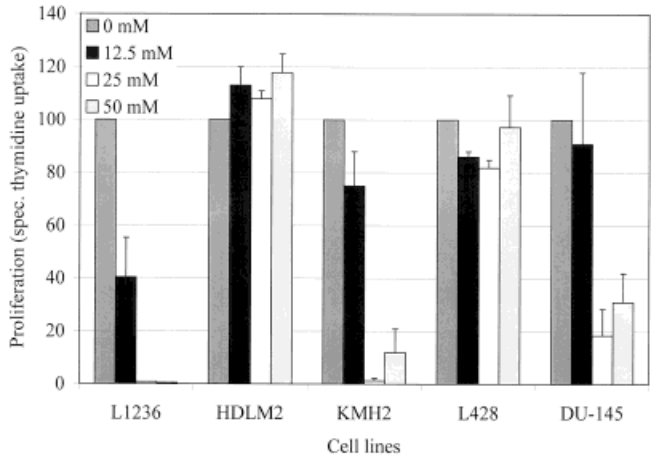

FIGURE 4 – Cyclocreatine inhibits cell proliferation in HD derived cell lines. Cell lines were incubated with cCr at the concentrations indicated, ^{3}H thymidine was added at Day 2 and uptake measured after 16 hr. Proliferation was quantified by calculating thymidine uptake in % from untreated controls. Values represent means ± SD. The data represent 3 independent experiments.

effect on proliferation of the L1236 cell line but, if anything, caused a small, variable reduction in cell growth both in L428 and DU-145 cells. Therefore, a growth promoting effect of creatine on the tumor cells tested was excluded.

Cyclocreatine inhibition of cell proliferation does not induce apoptosis

Many antitumor agents cause cell death by inducing apoptosis.[31] We studied apoptosis in cCr treated tumor cells with a flow cytometry based assay using annexinV labeled with fluorescein (Fig. 7). AnnexinV binds to phosphatidylserine residues exposed at the outer cell membrane 3–4 hr after induction of apoptosis. The Hodgkin-derived cell lines and DU-145 prostate carcinoma cells were incubated with cCr for 3 days and then tested for annexin binding. The apoptotic fraction in the L1236 cell line never exceeded 10% at all cCr concentrations used. With 25 or 50 mM cCr DU-145 cells exhibited an increase of apoptotic cells to 10% or 27%, respectively. Surprisingly, 38% of L428 cells underwent apoptosis at a cCr concentration of 50 mM despite not being affected by cCr in their proliferative capacity.

DISCUSSION

Differential display RT-PCR was applied to isolate mRNAs expressed in the Hodgkin-derived cell lines L1236, L428 or L540, but not in EBV transformed B-cells. We identified ubiquitous mitochondrial creatine kinase (uMtCK) to be overexpressed in several HD cell lines and analyzed its potential role as an antitumor target after incubation with the creatine analogue cyclocreatine.

The scarcity of H-RS cells, comprising only 0.1–1% of the involved tissue, prevented the use of primary tumor samples for DD RT-PCR. Instead, 3 well characterized cell lines derived from patients with classical HD were used. Two of them, L428[32] and L1236,[20] have rearranged Ig genes, *i.e.*, are B-cells as are the primary H-RS cells in most of the cases analyzed so far. Single cell PCR actually showed that the L1236 cell line, which has been established from the peripheral blood of a HD patient, has the same sequence of the Ig rearrangement as the H-RS cells in the patient's bone marrow and lymph node.[1] So far, this is the only cell line in which derivation from the patients H-RS cells has been proven. Two non-malignant EBV transformed B-cell lines (LCLs) were used as the normal counterpart for differential display. To avoid genes related to EBV infection we only isolated cDNAs from the HD panel and disregarded genes selectively expressed in LCLs.

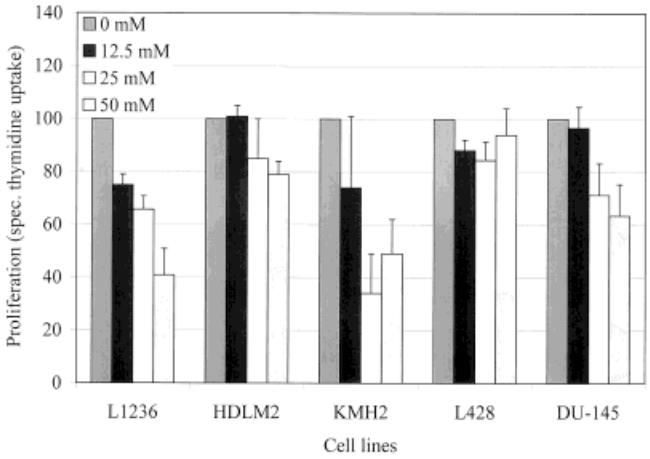

FIGURE 5 – A combination of cyclocreatine with creatine reduces the inhibitory effect of cyclocreatine. Cell lines were incubated with 50 mM creatine and cCr at the concentrations indicated, ^{3}H thymidine was added at Day 2 and uptake measured after 16 hr. Proliferation was quantified by calculating thymidine uptake in % from untreated controls. Values represent means ± SD. The data represent 3 independent experiments.

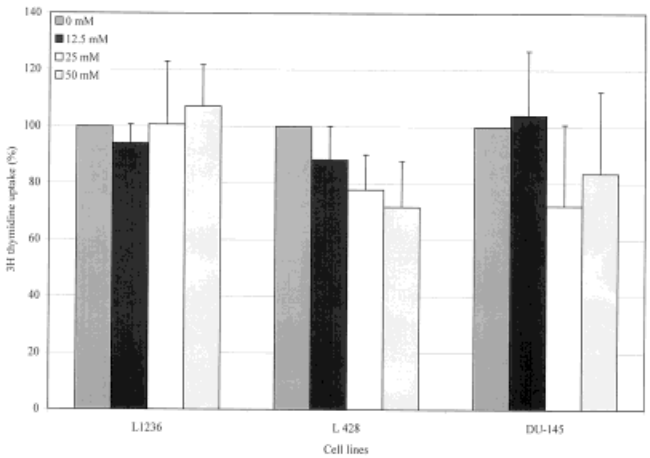

FIGURE 6 – Creatine itself does not promote tumor cell proliferation. Cell lines were incubated with creatine at the concentrations indicated, ^{3}H thymidine was added at Day 2 and uptake measured after 16 hr. Proliferation was quantified by calculating thymidine uptake in % from untreated controls. Values represent means ± SD. The data represent 3 independent experiments.

One of the 36 differentially expressed cDNAs isolated had a 100% homology to ubiquitous mitochondrial creatine kinase (uMtCK). We found uMtCK in 2 further HD lines using RT-PCR and could confirm the presence of uMtCK in the L1236 cell line by Western blot. CK activity was verified by a pH stat assay. Creatine kinase is usually expressed in cells with a high and fluctuating energy demand, such as brain or muscle cells.[4] In these cells, ubiquitous uMtCK or sarcomeric sMtCK is generally co-expressed with their respective cytosolic counterpart, B- or M-CK. In tumors, numerous studies have associated CK with growth, proliferation, motility and metastatic potential. In fact, CK is overexpressed in most tumors as compared to the corresponding non-malignant tissue.[6,8,33] Downregulation of B-CK in breast cancer correlates with growth inhibition[34] and elevated CK plasma levels are a diagnostic marker[35] correlating with aggressiveness of cancer cells.[36] Overexpression of mitochondrial CK was also described as a marker for poor prognosis.[6,8]

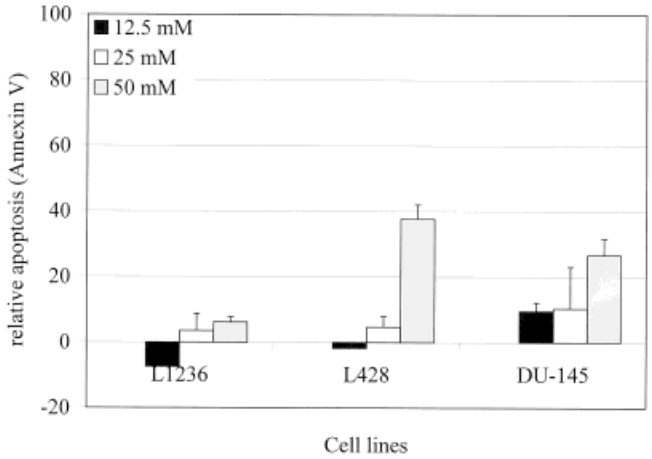

FIGURE 7 – Apoptosis in cCr-treated tumor cells. Apoptotic cells were detected by a flow cytometry based assay using AnnexinV labeled with fluorescein. Negative values reflect a lower percentage of apoptotic cells, positive values a higher percentage of apoptotic cells in the cCr-treated cells compared to untreated cells. Values represent means ± SD. The data represent 3 independent experiments.

Although CK expression is clearly associated with tumor growth, it is not yet clear how CK confers an advantage to tumor cells. Our results on the L1236 cell line, demonstrating overexpression of uMtCK and the absence of B-CK, M-CK and sMtCK, exclude the existence of a functional Cr/PCr energy shuttle between mitochondria and cytosol in this cell line. A similar conclusion was reached with antisense inhibition in HeLa cells, where uMtCK is coexpressed with B-CK. An 80% inhibition of uMtCK expression by an antisense oligonucleotide did not influence the proliferative capacity of these carcinoma cells.[37] A single CK isoform may be sufficient, however, to maintain an energy buffer function in tumor cells. For example, M-CK knock-out mice, expressing only sarcomeric MtCK, show normal levels of ATP and PCr and are able to use the PCr pool for ATP-regeneration during muscle exercise.[38] Similarly, transgenic mice expressing MtCK in liver, an organ that is normally devoid of any CK isoform, were producing and utilizing PCr in the liver tissue.[39] Apparently, if MtCK is missing its cytosolic counterpart, the MtCK reaction, that usually works in the direction of PCr synthesis, can be reversed to produce ATP. This is probably due to an increased permeability of the outer mitochondrial membrane for ADP and PCr.[40] With transgenic liver expressing B-CK, it was also shown that this tissue becomes tolerant against endotoxins, hypoxia and anoxia,[41,19] indicating that CK, together with its substrate Cr, can confer cell protection.

One approach for probing the metabolic role of CK in tumor tissue was to replace Cr with the analogue cyclocreatine (cCr). cCr impairs cellular energy metabolism by accumulating as phospho-cCr (PcCr), a very poor substrate for CK in the reverse reaction.[14] Thus, PcCr represents a trap for high energy phosphates in the cell. Our results with cCr provide 2 lines of evidence in favor of an energy buffering function of uMtCK in HD cells. First, in agreement with many other studies on transplanted solid tumors and tumor cell lines,[15,17] we have found an inhibitory effect of cCr only in those HD cell lines that express CK, especially pronounced in the L1236 cell line and, to a lesser degree, in cell line KMH2. Only HDLM2 cells were not responding to cCr treatment. Thus, the presence of CK activity and the conversion of cCr into its phospho-compound are necessary, although not sufficient for the antiproliferative action of cCr. Second, competition of cCr with Cr partially restores growth capability of CK-expressing HD cell lines. A similar competition by Cr has been observed for cCr-inhibition of tumor cell motility.[16] Taken together, these results suggest an impairment of energy metabolism as a primary target of

cCr inhibition and substantiate a role of uMtCK overexpression in energy buffering of HD cells.

A number of alternative mechanisms, that are independent of energy metabolism, have been proposed for the action of Cr and cCr. These include a decrease of intracellular pH,[18] inhibition of glycolytic enzymes,[16] stabilization of microtubules,[42] pancreatic hormone levels,[43] inhibition of progression out of all phases of the cell cycle,44 cotransport of Na^+ followed by an increased intracellular Na^+ concentration[45] and other unknown downstream effectors. Concerning our experiments, it may be argued that the relatively high cCr concentration (12.5–50 mM) could have a non-specific influence on cell growth, *i.e.*, by increasing the osmolarity of the culture medium. L428 cells, negative for CK, showed indeed a large increase of apoptosis at the highest cCr concentration. Despite this fact, however, the proliferative capacity of L428 cells was unimpaired and the other cell lines, sensitive to cCr treatment, had only a small fraction of apoptotic cells. In addition, the observed competition of cCr by Cr would argue for a rather specific mechanism.

Another property of MtCK that is particular to this isoform may yet play a role in tumor growth. The enzyme interacts functionally and possibly also structurally with the adenylate translocator (ANT) in the outer mitochondrial membrane and mitochondrial porin (VDAC) in the inner mitochondrial membrane.[11,46] Both transmembrane proteins are prominent components of the Ca^{2+}-induced mitochondrial permeability transition pore (PTP), whose opening triggers the execution pathway of apoptosis.[13] In fact, using isolated mitochondria, octameric MtCK was shown to delay or prevent the opening of PTP.[12] Thus, overexpression of uMtCK may well impede apoptotic elimination of cancer cells. In fact, tumor cells resistant to apoptosis may escape cell death induced by radiation, chemotherapy or cytolytic effector cells. This would also explain why overexpression of mitochondrial CK is associated with aggressive tumors and poor prognosis.[6,8]

Because the majority of anticancer agents induce apoptosis in sensitive tumor cells, we have studied whether this is also the case for cCr. Our experiments clearly show that the inhibitory activity of cCr is not related to an increase in apoptosis, *e.g.*, via a destabilization of the MtCK octamer. Indeed, in isolated mitochondria, cCr rather stabilized MtCK octamers and delayed PTP opening.[12] Treatment with cCr, causing significant growth inhibition independent of apoptosis, could therefore be especially advantageous in cells resistant to proapoptotic stimuli. The application of cCr as an anticancer agent[15,44] in combination with standard chemotherapy of cancers expressing CK activity has been shown *in vitro* as well as in animal models to greatly enhance the effects of a number of cytotoxic agents.[47] Thus, such a strategy may be worth to be tested in CK positive human lymphomas.

ACKNOWLEDGEMENTS

This work was supported in part by grants from Dr. Mildred Scheel Stiftung fuer Krebsforschung, Germany (to M.K.), the Swiss National Science Foundation (3100-50824.97 to U.S. and T.W.) and the Swiss Cancer League (to U.S. and T.W.).

REFERENCES

1. Kanzler H, Hansmann ML, Kapp U, et al. Molecular single cell analysis demonstrates the derivation of a peripheral blood-derived cell line (L1236) from the Hodgkin/Reed-Sternberg cells of a Hodgkin's lymphoma patient. Blood 1996;87:3429–36.
2. Henderson S, Rowe M, Gregory C, et al. Induction of bcl-2 expression by Epstein-Barr virus latent membrane protein 1 protects infected B cells from programmed cell death. Cell 1991;65:1107–15.
3. Weber-Matthiesen K, Deerberg J, Poetsch M, et al. Numerical chromosome aberrations are present within the CD30+ Hodgkin and Reed-Sternberg cells in 100% of analyzed cases of Hodgkin disease. Blood 1995;86:1464–8.
4. Wallimann T, Wyss M, Brdiczka D, et al. Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: the 'phosphocreatine circuit' for cellular energy homeostasis. Biochem J 1992;281:21–40.
5. DeLuca M, Hall N, Rice R, et al. Creatine kinase isozymes in human tumors. Biochem Biophys Res Commun 1981;99:189–95.
6. Kanemitsu F, Kawanishi I, Mizushima J, et al. Mitochondrial creatine kinase as a tumor-associated marker. Clin Chim Acta 1984;138:175–83.
7. Martin KJ, Chen SF, Clark GM, et al. Evaluation of creatine analogues as a new class of anticancer agents using freshly explanted human tumor cells. J Natl Cancer Inst 1994;86:608–13.
8. Pratt R, Vallis LM, Lim CW, et al. Mitochondrial creatine kinase in cancer patients. Pathology 1987;19:162–5.
9. Becker S, Schneider F. Investigations on the function of creatine kinase in Ehrlich ascites tumor cells. Biol Chem Hoppe Seyler 1989; 370:357–64.
10. Shimizu R. Clinical evaluation of mitochondrial creatine kinase levels in human tumors. Nippon Geka Hokan 1986;55:96–105.
11. Schlattner U, Forstner M, Eder M, et al. Functional aspects of the X-ray structure of mitochondrial creatine kinase: a molecular physiology approach. Mol Cell Biochem 1998;184:125–40.
12. O'Gorman E, Beutner G, Dolder M, et al. The role of creatine kinase in inhibition of mitochondrial permeability transition. FEBS Lett 1997;414:253–7.
13. Crompton M. The mitochondrial permeability transition pore and its role in cell death. Biochem J 1999;341:233–49.
14. Annesley TM, Walker JB. Cyclocreatine phosphate as a substitute for creatine phosphate in vertebrate tissues. Energistic considerations. Biochem Biophys Res Commun 1977;74:185–90.
15. Lillie JW, O'Keefe M, Valinski H, et al. Cyclocreatine (1-carboxymethyl-2-iminoimidazolidine) inhibits growth of a broad spectrum of cancer cells derived from solid tumors. Cancer Res 1993;53:3172–8.
16. Mulvaney PT, Stracke ML, Nam SW, et al. Cyclocreatine inhibits stimulated motility in tumor cells possessing creatine kinase. Int J Cancer 1998;78:46–52.
17. Schimmel L, Khandekar VS, Martin KJ, et al. The synthetic phosphagen cyclocreatine phosphate inhibits the growth of a broad spectrum of solid tumors. Anticancer Res 1996;16:375–80.
18. Kristensen CA, Askenasy N, Jain RK, et al. Creatine and cyclocreatine treatment of human colon adenocarcinoma xenografts: ^{31}P and ^{1}H magnetic resonance spectroscopic studies. Br J Cancer 1999;79:278–85.
19. Miller K, Halow J, Koretsky AP. Phosphocreatine protects transgenic mouse liver expressing creatine kinase from hypoxia and ischemia. Am J Physiol 1993;265:C1544–51.
20. Wolf J, Kapp U, Bohlen H, et al. Peripheral blood mononuclear cells of a patient with advanced Hodgkin lymphoma give rise to permanently growing Hodgkin-Reed Sternberg cells. Blood 1996;87:3418–28.
21. Nilsson K. Human B-lymphoid cell lines. Hum Cell 1992;5:25–41.
22. Liang P, Pardee AB. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science 1992;257: 967–71.
23. Drexler HG. Recent results on the biology of Hodgkin and Reed-Sternberg cells. II. Continuous cell lines. Leuk Lymphoma 1993;9:1–25.
24. Menezes J, Klein G, Clements G. Establishment and characterization of an Epstein-Barr virus (EBC)-negative lymphoblastoid B cell line (BJA-B) from an exceptional, EBV-genome-negative African Burkitt lymphoma. Biomedicine 1975;22:276–84.
25. Schneider U, Schwenk HU, Bornkamm G. Characterization of EBV-genome negative "null" and "T" cell lines derived from children with acute lymphoblastic leukemia and leukemic transformed non-Hodgkin lymphoma. Int J Cancer 1977;19:521–6.
26. Stone KR, Mickey DD, Wunderli H, et al. Isolation of a human prostate carcinoma cell line (DU 145). Int J Cancer 1978;21:274–81.
27. Lenoir GM, Vuillaume M, Bonnardel C. The use of lymphomatous and lymphoblastoid cell lines in the study of Burkitt lymphoma. IARC Sci Publ 1985;60:309–18.
28. Thalmeier K, Meissner P, Reisbach G, et al. Establishment of two permanent human bone marrow stromal cell lines with long-term post irradiation feeder capacity. Blood 1994;83:1799–807.
29. Schlattner U, Eder M, Dolder M, et al. Divergent enzyme kinetics and structural properties of the two human mitochondrial creatine kinase isoenzymes. Biol Chem 2000;381:1063–70.
30. Wallimann T, Schlosser T, Eppenberger HM. Function of M-line-bound creatine kinase as intramyofibrillar ATP regenerator at the receiving end of the phosphorylcreatine shuttle in muscle. J Biol Chem 1984;259:5238–46.

31. Bold RJ, Termuhlen PM, McConkey DJ. Apoptosis, cancer and cancer therapy. Surg Oncol 1997;6:133–42.
32. Vockerodt M, Soares M, Kanzler H, et al. Detection of clonal Hodgkin and Reed-Sternberg cells with identical somatically mutated and rearranged VH genes in different biopsies in relapsed Hodgkin disease. Blood 1998;92:2899–907.
33. Shatton JB, Morris HP, Weinhouse S. Creatine kinase activity and isozyme composition in normal tissues and neoplasms of rats and mice. Cancer Res 1979;39:492–501.
34. Kristensen CA, Kristjansen PE, Brunner N, et al. Growth inhibition in response to estrogen withdrawal and tamoxifen therapy of human breast cancer xenografts evaluated by *in vivo* ^{31}P magnetic resonance spectroscopy, creatine kinase activity and apoptotic index. Cancer Res 1995;55:4146–50.
35. Carney DN, Zweig MH, Ihde DC, et al. Elevated serum creatine kinase BB levels in patients with small cell lung cancer. Cancer Res 1984;44:5399–403.
36. Neri B, Bartalucci S, Romano S, et al. Evaluation of creatine kinase isoenzyme BB as a marker of neoplastic growth in Yoshida ascites hepatoma of the rat. Anticancer Res 1985;5:533–6.
37. Enjolras N, Godinot C. Inhibition of ubiquitous mitochondrial creatine kinase expression in HeLa cells by an antisense oligodeoxynucleotide. Mol Cell Biochem 1997;167:113–25.
38. van Deursen J, Heerschap A, Oerlemans F, et al. Skeletal muscles of mice deficient in muscle creatine kinase lack burst activity. Cell 1993;74:621–31.
39. Miller K, Sharer K, Suhan J, et al. Expression of functional mitochondrial creatine kinase in liver of transgenic mice. Am J Physiol 1997;272:C1193–202.
40. Veksler VI, Kuznetsov AV, Anflous K, et al. Muscle creatine kinase-deficient mice. II. Cardiac and skeletal muscles exhibit tissue-specific adaptation of the mitochondrial function. J Biol Chem 1995;270:19921–9.
41. Hatano E, Tanaka A, Iwata S, et al. Induction of endotoxin tolerance in transgenic mouse liver expressing creatine kinase. Hepatology 1996;24:663–9.
42. Martin KJ, Vassallo CD, Teicher BA, et al. Microtubule stabilization and potentiation of Taxol activity by the creatine analog cyclocreatine [published erratum appears in Anticancer Drugs 1995 Aug;6(4):618]. Anticancer Drugs 1995;6:419–26.
43. Ara G, Gravelin LM, Kaddurah-Daouk R, et al. Antitumor activity of creatine analogs produced by alterations in pancreatic hormones and glucose metabolism. In Vivo 1998;12:223–31.
44. Martin KJ, Winslow ER, Kaddurah-Daouk R. Cell cycle studies of cyclocreatine, a new anticancer agent. Cancer Res 1994;54:5160–5.
45. Maril N, Degani H, Rushkin E, et al. Kinetics of cyclocreatine and Na^+ cotransport in human breast cancer cells: mechanism of activity. Am J Physiol 1999;277:C708–16.
46. Stachowiak O, Schlattner U, Dolder M, et al. Oligomeric state and membrane binding behavior of creatine kinase isoenzymes: implications for cellular function and mitochondrial structure. Mol Cell Biochem 1998;184:141–51.
47. Teicher BA, Menon K, Northey D, et al. Cyclocreatine in cancer chemotherapy. Cancer Chemother Pharmacol 1995;35:411–6.