The MCT4 Gene: A Novel, Potential Target for Therapy of Advanced Prostate Cancer

Stephen Yiu Chuen Choi1,2, Hui Xue1,2, Rebecca Wu2, Ladan Fazli1, Dong Lin1,2, Colin C. Collins1, Martin E. Gleave1, Peter W. Gout2, and Yuzhuo Wang1,2

Abstract

Purpose: The management of castration-resistant prostate cancer (CRPC) is a major challenge in the clinic. Androgen receptor signaling–directed strategies are not curative in CRPC therapy, and new strategies targeting alternative, key cancer properties are needed. Using reprogrammed glucose metabolism (aerobic glycolysis), cancer cells typically secrete excessive amounts of lactic acid into their microenvironment, promoting cancer development, survival, and progression. Cellular lactic acid secretion is thought to be predominately mediated by MCT4, a plasma membrane transporter protein. As such, the MCT4 gene provides a unique, potential therapeutic target for cancer.

Experimental Design: A tissue microarray of various Gleason grade human prostate cancers was stained for MCT4 protein. Specific, MCT4-targeting antisense oligonucleotides (MCT4 ASO) were designed and candidate MCT4 ASOs checked for effects on (i) MCT4 expression, lactic acid secretion/content, glucose consumption, glycolytic gene expression, and proliferation of human CRPC cells and (ii) growth of PC-3 tumors in nude mice.

Results: Elevated MCT4 expression was associated with human CRPC and an earlier time to relapse. The treatment of PC-3, DU1145, and C4-2 CRPC cultures with candidate MCT4 ASOs led to marked inhibition of MCT4 expression, lactic acid secretion, to increased intracellular lactic acid levels, and markedly reduced aerobic glycolysis and cell proliferation. Treatment of PC-3 tumor-bearing nude mice with the MCT4 ASOs markedly inhibited tumor growth without inducing major host toxicity.

Conclusions: MCT4-targeting ASOs that inhibit lactic acid secretion may be useful for therapy of CRPC and other cancers, as they can interfere with reprogrammed energy metabolism of cancers, an emerging hallmark of cancer.

Introduction

Prostate cancer is the most commonly diagnosed noncutaneous cancer and a leading cause of cancer-related death for North American men (1). When the malignancy is confined to the prostate, surgery and radiotherapy can be curative. However, many treated patients experience local cancer recurrence and metastasis (2). Although androgen deprivation therapy (ADT), currently the treatment of choice for metastatic prostate cancer, can lead to remissions, tumors frequently return in a form that is highly resistant to ADT and other therapies, that is, metastatic castration-resistant prostate cancer (mCRPC). Although the efficacy of mCRPC treatment has recently been improved by using more powerful chemotheeraputics targeting the androgen receptor (AR) signaling axis, such as enzalutamide (3) and abiraterone (4), the overall survival of patients has only marginally increased (5–7). Moreover, it is thought that further improved versions of such drugs can promote transdifferentiation of prostatic adenocarcinoma to neuroendocrine prostate cancer, a subtype of the disease that is currently incurable (8–10). There is, therefore, a critical need for new, more effective strategies for therapy of CRPC that target different key properties of cancer.

There is increasing evidence that targeting reprogrammed energy metabolism of cancers offers a unique approach for effective therapeutic intervention (11). For glucose utilization, cancer cells, as distinct from normal resting cells, in general have a preference for glycolysis coupled to lactic acid production, i.e., a process called aerobic glycolysis (the Warburg effect; ref. 12). This leads to elevated glucose consumption, a near-universal property of primary and metastatic cancers. In addition, aberrant utilization of glutamine, also leading to elevated lactic acid production, has been observed to be highly common for cancers (13). These metabolic energy pathways lead to increased lactic acid secretion by the cancer cells into their microenvironment, facilitating multiple oncogenic, lactate-stimulated processes, including tissue invasion/metastasis, neoangiogenesis and responses to hypoxia (14–17); furthermore, lactic acid–induced acidification of the cancer cell microenvironment (to pH 6.0–6.5) can lead to the suppression of local host antitumor immunity (14, 18). The phenomenon of enhanced glucose metabolism by cancers is most commonly exploited clinically by 18F-fluoro-deoxyglucose PET (FDG-PET). Although this imaging technique is not generally used for prostate cancer (19), there is evidence suggesting that glucose metabolism of prostate cancer cells is increased by AR signaling and progression to treatment resistance (20, 21). As such, targeting the aerobic glycolytic pathway could be effective for treating advanced prostate cancers.
Translational Relevance
The management of castration-resistant prostate cancer (CRPC) is a major challenge in the clinic. Although CRPC treatment efficacy has been improved by using more powerful drugs targeting the androgen receptor signaling axis, patient survival has only marginally increased, and further improved versions of such drugs can lead to transdifferentiation of CRPC to currently incurable neuroendocrine prostate cancer. Therefore, new strategies targeting alternative, fundamental cancer properties are critically needed. Increasing evidence indicates that reprogrammed energy metabolism of cancers offers a unique target. For glucose utilization, cancer cells generally prefer glycolysis coupled to lactic acid production, that is, aerobic glycolysis. This pathway leads to increased lactic acid secretion by cancers facilitating oncogenic processes, including tissue invasion/metastasis and neoangiogenesis. The lactic acid secretion is predominantly mediated by MCT4, a plasma membrane transporter protein. Here, we have shown, both in vitro and in vivo, that MCT4-targeting antisense oligonucleotides (ASO) can inhibit MCT4 expression, lactic acid secretion, aerobic glycolysis, and cell proliferation of CRPC models. As such, MCT4-targeting ASOs may provide a novel therapeutic approach for CRPC.

The monocarboxylate transporter (MCT) family consists of plasma membrane transporter proteins involved in the transport of lactic acid and other metabolic monocarboxylates. In particular, the cellular efflux of lactic acid/H+ is thought to be predominantly mediated by MCT4 (SLC16A3; ref. 22). The expression of MCT4 has been associated with highly glycolytic cells (17,23,24), and elevated expression of MCT4 in tumors is associated with highly glycolytic prostate cancer (28,29). Furthermore, elevated MCT4 expression has been associated with highly glycolytic prostate cancer (28,29). In this study, we established that the development of human CRPC is associated with elevated expression of MCT4. Furthermore, evidence was obtained that MCT4-targeting antisense oligonucleotides (ASO) inhibiting MCT4-mediated lactic acid secretion may be useful for the treatment of CRPC.

Materials and Methods

Materials
Chemicals, solvents, and solutions were obtained from Sigma-Aldrich, unless otherwise indicated.

Cell cultures
Human PC-3 and DU145 CRPC cells, human LNCaP prostate cancer cells, and mouse TRAMPc2 prostate cancer cells were purchased from the ATCC. C4-2 CRPC cells were obtained from Dr. Martin E. Gleave (Vancouver Prostate Centre, Vancouver, British Columbia, Canada). Human monolayer cultures were maintained in RPMI1640 (GE Healthcare HyClone), supplemented with 10% FBS (GE Healthcare HyClone), whereas TRAMPc2 cells were maintained in DMEM (GE Healthcare HyClone), supplemented with 5% FBS. For cell counting, cells were trypsinized to form a single-cell suspension and counted using a TC20 Automated Cell Counter (Bio-Rad). Cell viability was assessed by Trypan blue exclusion.

Human prostate cancer TMA construction and IHC
Tissue microarrays (TMA) were manually constructed, as previously described (37,38), using various Gleason grades–exhibiting prostate cancer specimens (n = 342), obtained from the Vancouver Prostate Centre Tissue Bank with written informed consent of patients, clinical information, and institutional study approval. All specimens were obtained through radical prostatectomy, except CRPC samples that were obtained via transurethral resection of the prostate. Immunohistochemical staining was conducted using a Ventana autostainer (model Discover XT, Ventana Medical Systems) with an enzyme-labeled biotin–streptavidin system and a solvent-resistant DAB Map Kit (Ventana Medical Systems). Staining intensity was scored by a trained pathologist on a 4-point scale: 0 represents no staining on any tumor cells, 1 represents a faint or focal, questionably present stain, 2 represents a stain of convincing intensity in a minority of cells, and 3 represents a stain of convincing intensity in a majority of cells.

Antibodies
The following antibodies and conjugates were used: rabbit anti-MCT4 antibody (Santa Cruz Biotechnology; WB 1:4,000, IHC 1:100), mouse anti-vinculin antibody (Sigma; WB 1:1,000), rabbit anti-cleaved caspase-3 antibody (Cell Signaling Technology; IHC 1:50), mouse anti-Ki67 antibody (Dako; IHC 1:50), rat anti-CD31 antibody (Dianova; IHC 1:20), mouse anti-pan-T cell marker CD3 antibody (Dako; IHC 1:50), biotinylated mouse anti-NK1.1 antibody (Cedarlane; IHC 1:100), IRDye 800CW goat anti-mouse antibody (LI-COR Biosciences; WB 1:10,000), IRDye 680RD goat anti-rabbit antibody (LI-COR Biosciences; WB 1:10,000), biotinylated goat anti-rabbit antibody (Vector Laboratories; IHC 1:200), biotinylated goat anti-rat antibody (Vector Laboratories; IHC 1:200), and biotinylated goat anti-mouse antibody (Vector Laboratories; IHC 1:200).

ASO design and selection
First-generation phosphorothioate-modified ASOs against human MCT4 were rationally designed by selecting sequences containing favorable motifs while excluding unfavorable ones (39). Specificity of MCT4-targeting sequences, compared with human and mouse genes (at least 3/20 bases mismatched), was evaluated using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Ten sequences (see Supplementary Table S1) distributed throughout the length of the transcript with perfect complementarity to all human MCT4 transcript variants (NM_001042422.2, NM_001042423.2, NM_001206950.1, NM_001206951.1, NM_001206952.1, and NM_004207.3) were selected and synthesized by Eurofins MWG Operon. The
MCT4 staining of CRPC TMAs reveals elevated MCT4 expression as clinically relevant. TMAs were constructed using patient-derived prostate cancer samples available at the Vancouver Prostate Centre Tissue Bank and stained for MCT4 expression. A, results confirm that elevated MCT4 expression is associated with high Gleason grade. B, representative images of MCT4 staining show increased staining intensity in Gleason grade 5 specimens. C, high MCT4 expression is associated with earlier time to relapse as measured by increases in serum PSA. D, patients undergoing prolonged neoadjuvant hormone therapy (NHT) and those with CRPC also show elevated MCT4 expression. E, representative images of MCT4 staining show an increase in staining intensity in extended neoadjuvant hormone therapy and CRPC patient specimens. *, P < 0.05; **, P < 0.01.

Figure 1.
MCT4 staining of CRPC TMAs reveals elevated MCT4 expression as clinically relevant. TMAs were constructed using patient-derived prostate cancer samples available at the Vancouver Prostate Centre Tissue Bank and stained for MCT4 expression. A, results confirm that elevated MCT4 expression is associated with high Gleason grade. B, representative images of MCT4 staining show increased staining intensity in Gleason grade 5 specimens. C, high MCT4 expression is associated with earlier time to relapse as measured by increases in serum PSA. D, patients undergoing prolonged neoadjuvant hormone therapy (NHT) and those with CRPC also show elevated MCT4 expression. E, representative images of MCT4 staining show an increase in staining intensity in extended neoadjuvant hormone therapy and CRPC patient specimens. *, P < 0.05; **, P < 0.01.
knockdown efficiencies of these ten ASOs were tested by determining target mRNA and protein expression 48 hours after transfection of cells using qPCR and Western blotting. Two candidate ASOs (#1 and #14) were selected for further studies. Sequences: ASO #1, 5'-TCCCATGGCAGGAGGTTGC-3'; ASO #14, 5'-AGATGCAAGAACCCAGACC-3'; a published nontargeting control ASO, 5'-CCITCCCTGAAGGTTCCTCC-3' (40,41).

**ASO and siRNA transfection**

Cells were transfected in 6-well plates with ASOs at 100 nmol/L for 48 hours (unless otherwise indicated) using Oligofectamine (Invitrogen) or with MCT4-targeting siRNAs and controls (Dharmacon) at 50 nmol/L for 48 hours using Lipofectamine 2000 (Invitrogen), following the manufacturer's instructions.

**Quantitative PCR**

Total RNA was isolated using the RNeasy Mini Kit (Qiagen Inc.) and cdNA synthesized using the Quant iTect Reverse Transcription Kit (Qiagen). Primers (see Supplementary Table S1) were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). qRT-PCR reactions using KAPA SYBR FAST Universal (Kapa Biosystems) were performed in triplicate in a 96-well Real-Time PCR System (Applied Biosystems). Target genes were normalized to a geometric average of 3 internal reference genes (42).

**Western blotting**

Cells were harvested and lysed in RIPA buffer (50 mmol/L Tris Cl pH 7.4, 150 mmol/L NaCl, 1% IGEPA, 0.5% sodium deoxycholate, and 0.1% SDS), supplemented with a complete protease inhibitor cocktail (Roche). The protein concentration of the lysate was determined by Pierce BCA Protein Assay (Thermo Scientific). Cells were lysed in sodium cholate, and 0.1% SDS), supplemented with a complete protease inhibitor cocktail (Roche) or with MCT4-targeting siRNAs and controls (Dharmacon) at 50 nmol/L for 48 hours using Lipofectamine 2000 (Invitrogen), following the manufacturer's instructions.

**IHC of tumor tissue**

Tumor tissue was formalin-fixed and paraffin-embedded for immunohistochemical analysis. Tissues were sectioned, probed, and stained with DAB (Sigma) as previously described (43). For Ki-67 and cleaved caspase-3 staining, images of five random fields at 400x magnification were taken per tumor and cells counted to determine the percentage of positively stained cells. For MCT4, images of five random fields at 200x magnification were taken per tumor, and staining intensity was assessed by percentage scoring, using the formula: intensity = (% area score 3) × 3 + (% area score 2) × 2 + (% area score 1) × 1. The extent of immune cell aggregation was quantified following CD31 staining by imaging the five most prominent regions of aggregates per tumor at 200x magnification and determining the percent area of the field they occupied. The proportions of immune cells were evaluated as the area of positive staining normalized to the area occupied by immune cell aggregates in the same five prominent regions.

**Statistical analysis**

All pooled results are represented as mean ± SEM. Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, Inc). The Student t test was carried out to compare means between two groups. One-way ANOVA followed by the post hoc Dunnett test was used to compare means of more than two groups. Two-way ANOVA followed by post-hoc multiple comparison was applied to compare tumor growth. A contingency test was done to compare staining intensity among patient cohorts on the TMA. A log rank test was done to compare patient survival curves. 2x2 tests were done to correlate MCT4 expression levels with various clinical parameters. Results with P < 0.05 were considered statistically significant and are indicated by *, P < 0.01; and ***, P < 0.001.

**Results**

**Elevated MCT4 protein expression is associated with CRPC**

A TMA composed of tissues from Gleason grade 3, 4, and 5 human prostate cancers was stained for MCT4 protein. As shown...
Figure 2.
Efficacy screening of MCT4-targeting ASOs using PC-3 cells in vitro reveals candidates markedly inhibiting MCT4 expression and cell proliferation. MCT4 ASOs #1 and #14 exert their in vitro effects in a dose-dependent manner, and the effects persist past 96 hours after transfection. A, siRNA silencing of MCT4 in PC-3 cells showed significant inhibition of cell proliferation, indicating that inhibition of MCT4 expression could have potential therapeutic efficacy. B, screening of ten MCT4-targeting ASOs revealed varying inhibitory effects on cell proliferation, with sequences #1 and #14 showing the most profound inhibitions. C, MCT4 ASOs induced various levels of MCT4 knockdown as measured by qPCR and Western blot, with ASOs #1 and #14 being the most effective. D, a strong correlation ($P < 0.001$) was found between the MCT4 mRNA expression and the resulting cell numbers, indicating that the inhibitory effects on cell proliferation are strongly related to decreased MCT4 expression (dotted lines represent the 95% confidence interval). E, transfection of 5 to 200 nmol/L of MCT4 ASOs showed that the inhibition of cell proliferation and expression of MCT4 is similarly dose dependent ($IC_{50} = 32$ nmol/L for ASO #14 and $IC_{50} = 50$ nmol/L for ASO #1). F, a time course experiment demonstrates that the inhibition of cell proliferation and MCT4 expression following MCT4 ASO transfections persists up to at least 96 hours post transfection, $***$, $P < 0.001$. 
Figure 3.
MCT4 ASOs #1 and #14 are effective in vitro against human prostate cancer cells other than PC-3, but not against mouse prostate cancer cells. A, candidate MCT4 ASOs are also able to inhibit C4-2 human prostate cancer cell proliferation and expression of MCT4 in a dose-dependent manner with IC50 values comparable to those observed with PC-3 cells. B, candidate MCT4 ASOs also inhibit DU145 human prostate cancer cell proliferation in a dose-dependent manner with IC50 values similar to those obtained with the other cell lines. The inhibition of cell proliferation is accompanied by a decrease in MCT4 expression. C, candidate MCT4 ASOs do not have any appreciable effects on TRAMPC2 mouse prostate cancer cells. They neither affect cell proliferation nor mouse MCT4 expression levels even at the highest tested concentration of 200 nmol/L. This indicates that our candidate MCT4 ASOs are specific for human MCT4 and that the inhibition of cell proliferation is a phenomenon related to MCT4 knockdown.
in Fig. 1A and B, Gleason grade 5 prostate cancers had significantly increased MCT4 protein expression relative to Gleason grade 3 and 4 specimens. Also, elevated MCT4 expression was associated with an earlier time to relapse from primary treatment, as measured by increases in serum prostate-specific antigen (PSA) levels, with the high MCT4–expressing cohort having a median time to relapse of 63.3 months versus 94.2 months for the low MCT4–expressing cohort (Fig. 1C). In addition, high MCT4 expression was correlated with other clinical characteristics associated with poor prognosis, such as higher serum PSA levels at diagnosis and clinical T stage (Supplementary Table S2). Furthermore, elevated MCT4 protein expression was found in tumors from patients subjected to prolonged neoadjuvant hormone therapy (>6 months) and CRPC patients (Fig. 1D and E), indicating that elevated expression of MCT4 protein in prostate cancer is associated with the development of CRPC.

Knockdown of MCT4 inhibits PC-3 cell proliferation

The potential therapeutic efficacy of inhibiting MCT4 expression was investigated using MCT4-targeting siRNAs and ASOs. Human PC-3 CRPC cells were used as they present a distinct glycolytic metabolic profile, a property associated with MCT4 expression (20). As shown in Fig. 2A, treatment of PC-3 cells with MCT4 siRNA led to an inhibition of cell proliferation, suggesting potential therapeutic efficacy of an MCT4 knockdown approach. Accordingly, ASOs specifically targeting human MCT4 were designed. Screening of ten MCT4 ASOs revealed varying capacities of inhibiting PC-3 cell proliferation and MCT4 expression, with ASO #1 and #14 showing the greatest potency (Fig. 2B and C). A strong correlation was found between the reduced levels of MCT4 mRNA in PC-3 cells treated with the various ASOs and the resulting cell numbers (Fig. 2D), indicating that the growth inhibition by the ASOs was directly related to MCT4 knockdown.

Candidate MCT4 ASOs inhibit PC-3 cell proliferation in a sustained and dose-dependent manner

The growth-inhibitory activities of MCT4 ASOs #1 and #14 were further characterized. As shown in Fig. 2E, PC-3 cell proliferation was inhibited by both ASOs in a dose-dependent manner, with ASO #14 being slightly more effective (IC_{50} = 26 nmol/L) than ASO #1 (IC_{50} = 50 nmol/L). The ASOs also reduced MCT4 mRNA levels in a dose-dependent manner, mirroring their inhibition of cell proliferation (IC_{50} ASO #14 = 32 nmol/L and IC_{50} ASO #1 = 50 nmol/L). As shown in Fig. 2F, both ASOs maintained the inhibition of cell proliferation even at 96 hours post transfection. While MCT4 mRNA levels began to increase slightly after 48 hours of transfection, MCT4 protein levels remained low even at 96 hours.

Candidate MCT4 ASOs exert similar growth-inhibitory and MCT4 expression–inhibitory effects in other human prostate cancer cell lines and are specific for human MCT4

When the MCT4 ASOs #1 and #14 were transfected into human C4-2 CRPC cells, they inhibited their proliferation with IC_{50} values comparable to those observed with PC-3 cells, i.e., ASO #1 = 40 nmol/L and ASO #14 = 27 nmol/L. Similarly, they reduced the MCT4 expression with IC_{50} values of 50 nmol/L for ASO #1 and 26 nmol/L for ASO #14 (Fig. 3A). Furthermore, when the ASOs were transfected into human DU145 prostate cancer cells, a similar inhibitory effect on cell proliferation and MCT4 expression was observed with almost identical IC_{50} values (Fig. 3B). Transfection of ASOs into LNCaP prostate cancer cells also showed a similar inhibition of cell proliferation and MCT4 expression (Supplementary Fig. S1), suggesting that the inhibitory effect is more associated with a glycolytic phenotype than androgen receptor status. Importantly, transfection of MCT4 ASOs #1 and #14 into mouse TRAMPc2 prostate cancer cells did not lead to a significant reduction in cell proliferation or mouse MCT4 expression (Fig. 3C). Taken together, the results suggest that these ASOs specifically target human MCT4 and that their inhibitory effect on cell proliferation is a consequence of MCT4 knockdown.

Candidate MCT4 ASOs are able to inhibit glucose metabolism and tissue invasion/migration of CRPC cells in vitro

To further examine the effects of MCT4-targeting ASOs on prostate cancer cells, we measured their effects on lactic acid secretion, intracellular lactate concentrations, and glucose consumption of PC-3 cells. Transfection of the cells with MCT4 ASOs #1 and #14 led to a marked inhibition of lactic acid secretion, a corresponding accumulation of intracellular lactate, and an extensive decrease in glucose consumption, measured after 48 hours of transfection (Fig. 4A). Furthermore, as shown in Fig. 4B, treatment with the ASOs resulted in the downregulation of various genes involved in glycolysis, i.e., GAPDH, PGK1, PGAM1, and ENO1. In addition, the expression of lactate dehydrogenase A was found to be depressed, indicative of a decrease in the conversion of pyruvate to lactic acid. Moreover, decreased expression was found for pyruvate dehydrogenase kinase-1, an enzyme that shunts pyruvate away from the TCA cycle and promotes its conversion to lactic acid. Thus, the treatment with the MCT4 ASOs led to inhibition of aerobic glycolysis.

Treatment with MCT4 ASOs also inhibited the migration and tissue invasion of PC-3 cells in modified Boyden chambers (Supplementary Fig. S2), suggesting that lactic acid secretion as facilitated by MCT4 could also play an important role in the metastatic process.

Growth of PC-3 xenografts in nude mice inhibited by treatment with MCT4 ASOs

Male athymic nude mice bearing subcutaneous PC-3 tumors were treated with MCT4 ASOs #1 and #14 for a total of 15 days. Both ASOs markedly inhibited the growth of the tumors (Fig. 5A) without inducing major host toxicity, as assessed by monitoring animal weights (Supplementary Fig. S3) and behavior. Immunohistochemical analysis revealed that the ASO-induced inhibition of tumor growth was associated with an increase in cell apoptosis, as measured by cleaved caspase-3 staining and a decrease in cell proliferation, as measured by Ki-67 staining (Fig. 5B). The decrease in tumor growth was associated with a decrease in MCT4 protein expression (Fig. 5C), consistent with an antiproliferative effect generated by MCT4 knockdown.

Effects of MCT4 ASOs on immune cell aggregates in nude mice

As lactic acid–induced acidification of tumors has been linked to the suppression of local host antitumor immunity (14), it was of interest to determine whether the treatment of the PC-3 tumor–
Figure 4.
Transfection of candidate MCT4 ASOs leads to the inhibition of glucose metabolism of PC-3 cells. A, MCT4 ASOs significantly inhibited lactic acid secretion. A corresponding accumulation of intracellular lactate and inhibition of glucose consumption were also observed 48 hours post transfection. u.d., undetectable. B, the changes in metabolism caused by MCT4 knockdown were further characterized by qPCR analysis of gene expression levels of various genes involved in glycolysis and lactic acid conversion. Following treatment with MCT4 ASOs, a marked change in glucose metabolism was observed in PC-3 cells. A significant decrease in expression of various genes in glycolysis suggests an overall decrease in glucose metabolism. Furthermore, the decreased expression of lactate dehydrogenase A (LDHA) and pyruvate dehydrogenase kinase-1 (PDK1) suggests a redirection of pyruvate away from lactic acid production toward the TCA cycle for oxidative phosphorylation. *, P < 0.05; **, P < 0.01; ***, P < 0.0001.
Figure 5.
MCT4 ASO-induced reduction of MCT4 expression in PC-3 tumor cells in vivo was associated with inhibition of PC-3 tumor growth, characterized by an increase in apoptosis and inhibition of cell proliferation. Athymic nude mice bearing subcutaneous PC-3 tumors were treated with intraperitoneal injections of MCT4 ASOs #1, #14, control ASO, or vehicle (PBS) at 10 mg/kg daily for 5 days followed by 2 days off treatment for a total of 15 days. A, treatment with the MCT4 ASOs significantly slowed down tumor growth. B, the decrease in tumor growth rates following treatment with MCT4 ASOs is attributable to an increase in cell apoptosis and a decrease in cell proliferation. C, treatment with MCT4 ASOs decreased MCT4 expression in the tumor, as measured by immunohistochemical staining. Representative images of tumors from each group show the presence of strong membrane staining in the control tumors that is absent in the MCT4 ASO-treated tumors. *, P < 0.05; **, P < 0.01.
bearing nude mice with MCT4 ASOs had caused changes in the local host immune response of these mice, even though their immune reactivity was very limited. To that end, we quantified immune cell aggregates that had extravasated from CD31+ blood vessels, particularly in the tumor periphery. As shown in Fig. 6A, xenografts treated with the two MCT4 ASOs had significantly larger immune cell aggregates compared with control tumors. Quantification of the natural killer (NK) cell population, the predominant cytotoxic immune cell subtype in nude mice (44), revealed that the treatment with the MCT4 ASOs markedly increased the proportion of tumor-associated NK cells (Fig. 6B). Furthermore, the activation of NK cells is facilitated by CD3 (45), a molecule commonly regarded as a T-cell marker for its association with the T-cell receptor complex. Its expression in NK cells is detectable by IFIC (46), and in view of the absence of T cells in nude mice, can be used as an indicator of NK cell activation (47). As shown in Fig. 6C, CD3 staining revealed that the proportion of activated NK cells associated with the ASO-treated tumors had also increased.

Discussion

Cancers have a common preference for reprogrammed energy metabolism leading to upregulated glycolysis and excessive lactic acid secretion, an emerging hallmark of cancer (48); the lactic acid secretion is thought to be predominantly mediated by the MCT4 plasma membrane transporter protein (22). This study indicates that the progression of prostate cancer to CRPC, a major challenge in the management of the disease (25,27), is coupled to an increase in MCT4 expression. Elevated MCT4 protein expression in clinical prostate cancer specimens was associated with increases in Gleason grade, prolonged treatment of patients with neoadjuvant hormone therapy, CRPC, and early disease relapse (Fig. 1A–E), as well as other clinicopathologic characteristics indicative of poor prognosis (Supplementary Table S2). These observations are consistent with reports by others (28,29), indicating that elevated MCT4 expression is clinically relevant and may play an important role in the late and more aggressive stages of the disease. Thus, stimulation of cancer progression by elevated expression of MCT4 leading to increased lactic acid secretion is consistent with the notion that lactic acid generated by cancers can aid their progression by promoting a variety of oncogenic processes.

Support for potential use of MCT4-targeting ASOs for therapy of CRPC comes from our findings that the treatment with MCT4 ASOs #1 and #4 markedly reduced MCT4 mRNA expression (Fig. 2C), lactic acid secretion, and glucose consumption by PC-3 cells (Fig. 4A) and inhibited in vitro proliferation of CRPC cell lines in a species-specific manner (Figs 2A–F and 3A–C). Furthermore, treatment of PC-3 tumor-bearing nude mice with the MCT4-targeting ASOs led to marked inhibition of the growth of the tumors (Fig. 5A), which was associated with increased apoptosis (Fig. 5B) and a reduction in MCT4 protein levels (Fig. 5C). As well, the treatment with the ASOs did not induce major toxicity to the hosts (Supplementary Fig. S3).

The MCT4 ASOs produced in this study appear to be highly specific for human MCT4, as the specificities of their sequences were checked via BLAST analysis (see Materials and Methods) and as they had highly similar inhibitory effects on the MCT4 expression and proliferation of human PC-3, DU145, C4-2, and LNCaP prostate cancer cells (Figs 2A–F, 3A and B, and Supplementary Fig. S1) as distinct from mouse TRAMPc2 prostate cancer cells, which were not significantly affected by the ASOs (Fig. 3C). The marked MCT4 ASO–induced inhibition of lactic acid secretion and corresponding increase in intracellular lactic acid concentration are therefore likely a direct result of MCT4 knockdown (Fig. 4A). The ASO-induced inhibition of glucose consumption (Fig. 4A) and downregulation of enzymes involved in aerobic glycolysis (Fig. 4B) appear to be secondary effects, probably reflecting a response of the cancer cells to reduce the potentially fatal increase in intracellular lactic acid content by shutting off the lactic acid supply. Furthermore, decreasing MCT4 expression also resulted in the reduction of Matrigel invasion and in vitro migration of PC-3 cells (Supplementary Fig. S2), lending preliminary confirmation that lactic acid secretion may play an important role in local tissue invasion and metastasis (15,16). Elucidation of the precise mechanisms by which the downregulation of MCT4 inhibits proliferation, tissue invasion, and migration of prostate cancer cells and other aspects of cancer biology, such as tumorigenesis, requires further studies. In particular, FDG-PET could be used as a metabolic tracer to determine whether glucose uptake by prostate cancer cells in vivo is affected by treatment with MCT4 ASOs. Alternatively, metabolomic approaches using stable isotope–labeled metabolites, such as 13C-glucose (49,50), may be used to more comprehensively assess alterations in glucose metabolism following MCT4 knockdown.

Of interest are the findings that the treatment of the PC-3 tumor–bearing nude mice with the MCT4 ASOs led to increases in (i) the number of extravasated immune cell aggregates in the tumor cell microenvironment (Fig. 6A), (ii) the proportions of host NK cells (Fig. 6B), and (iii) the proportions of host NK cells that had been activated (Fig. 6C). They indicate that MCT4 knockdown might lead to restoration of cancer-suppressed host immunity, a possibility that should be examined using immunocompetent hosts.

Elevated MCT4 expression has also been reported as clinically relevant and functionally significant in other types of cancer (25,27). For example, MCT4-targeting siRNA has been reported to be effective at inhibiting renal cell carcinoma growth (51), and MCT4 expression in small cell lung cancer was found to confer resistance to specific MCT1 inhibitors (32). The candidate MCT4 ASOs developed in our laboratory could therefore be useful in multiple clinical settings, in particular if their efficacies can be improved by incorporating second-generation 2′-methoxyethyl modifications in their backbone (52). In addition, treatment efficacy could also be increased using a combination strategy with other therapeutics currently used for treating CRPC (such as second-generation hormone therapies or docetaxel). Alternatively, combination with other modulators of glucose metabolism (such as metformin or mitochondrial inhibitors) might result in a more complete blockade of glucose utilization by cancer cells.

Conclusions

The development of CRPC was found to be associated with elevated expression of MCT4, a plasma membrane transporter protein mediating lactic acid secretion by cancers. MCT4-targeting ASOs that inhibit lactic acid secretion may be useful for therapy of CRPC and other cancers characterized by
Figure 6. MCT4-targeting ASOs increase extravasated immune cell aggregation and alter tumor-associated immune cell proportions in vivo. The potential immunomodulatory properties of MCT4 ASOs were investigated through immunohistochemical staining. A, immune aggregates are characterized by areas of small, circular, densely packed nuclei that are distinct from the surrounding tumor cells. Staining for CD31 reveals that these immune cells have extravasated and surround the blood vessels in the tumor periphery. The immunomodulatory effects of treatment with MCT4 ASO are partially exerted through a significant increase in the extent of such immune cell aggregations. B, treatment with MCT4 ASO also significantly altered the composition of the immune cells present in the aggregates. Staining using the NK cell marker NK1.1 revealed that treatment with MCT4 ASO increased the proportion of NK cells associated with the tumor. C, because nude mice lack T cells, CD3 was used as a marker for activated NK cells. Staining using CD3 also revealed that treatment with MCT4 ASO also increased the proportion of activated NK cells associated with the tumor, suggesting stimulation of anticancer immunity. *, P < 0.05; **, P < 0.01.
excessive lactic acid secretion resulting from reprogrammed cellular energy (glucose) metabolism, an emerging hallmark of cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: S.Y. Choi, C.C. Collins, M.E. Gleave, Y. Wang
Development of methodology: S.Y. Choi, H. Xue
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.Y. Choi, H. Xue, R. Wu, C.C. Collins, M.E. Gleave
Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): S.Y. Choi, D. Lin, C.C. Collins, M.E. Gleave
Writing, review, and/or revision of the manuscript: S.Y. Choi, C.C. Collins, M.E. Gleave, P.W. Gout, Y. Wang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Wu, D. Lin

References

Study supervision: C.C. Collins, Y. Wang
Other (pathology): L. Fazi

Acknowledgments
The authors thank James Killam, Fang Zhang, and Francesco Crea for their support during various scientific discussions and Estelle Li, Xindy Dong, Joy Wang, and Kendra Fu for their technical assistance.

Grant Support
This study was supported by The Urology Foundation for LAST Project (to Y. Wang), CIHR Master’s Award (Frederick Banting and Charles Best Canada Graduate Scholarships); to S.Y.C. Choi.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 8, 2015; revised November 30, 2015; accepted December 6, 2015; published OnlineFirst January 11, 2016.

www.aacrjournals.org Clin Cancer Res; 22(11) June 1, 2016 2733

MCT4 as a Potential Therapeutic Target for CRPC

on October 23, 2017. © 2016 American Association for Cancer Research.
The MCT4 Gene: A Novel, Potential Target for Therapy of Advanced Prostate Cancer

Stephen Yiu Chuen Choi, Hui Xue, Rebecca Wu, et al.


Updated version: Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-15-1624

Supplementary Material: Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2016/01/09/1078-0432.CCR-15-1624.DC1

Cited articles: This article cites 52 articles, 17 of which you can access for free at: http://clincancerres.aacrjournals.org/content/22/11/2721.full#ref-list-1

E-mail alerts: Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions: To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions: To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.