Keywords: multiple myeloma; glycolysis; PDK1; apoptosis

PDK1 inhibition is a novel therapeutic target in multiple myeloma

S Fujiwara¹, Y Kawano¹, H Yuki¹, Y Okuno¹, K Nosaka², H Mitsuya¹ and H Hata^{*,1,3}

¹Department of Hematology, Kumamoto University School of Medicine, 1-1-1 Honjo, Kumamoto 860-8556, Japan; ²Division of Chemotherapy, Kumamoto University School of Medicine, 1-1-1 Honjo, Kumamoto 860-8556, Japan and ³Division of Informative Clinical Science, Kumamoto University School of Medicine, 4-24-1 Kuhonji, Kumamoto 862-0976, Japan

Background: Cancer cells utilise the glycolytic pathway even when adequate oxygen is present, a phenomenon known as the Warburg effect. We examined whether this system is operative in multiple myeloma (MM) cells and whether glycolysis inhibition is a potential therapeutic modality.

Methods: The MM cells were purified from 59 patients using CD138-immunomagnetic beads. The expression levels of genes associated with glycolysis, c-MYC, GLUT1, LDHA, HIF1A and pyruvate dehydrogenase kinase-1 (PDK1) were determined by real-time PCR. Glucose consumption and lactate production by MM cell lines were analysed. Oxamate, an LDH inhibitor, and dichloroacetate (DCA), a PDK1 inhibitor, were employed. Inhibition of PDK1 expression was achieved using a siRNA.

Results: High LDHA expression was found to be an indicator of poor prognosis. It was also positively correlated with the expression of PDK1, c-MYC and GLUT1. Greater glucose consumption and lactate production in MM cells was associated with higher LDHA expression. All the glycolysis inhibitors (oxamate, DCA and PDK1 siRNA) induced apoptosis in MM cells. DCA combined with bortezomib showed additive cytotoxic effects.

Conclusion: The present data suggest that the Warburg effect is operative in MM cells. As PDK1 is not overexpressed in normal tissues, PDK1 inhibition could serve as a novel therapeutic approach.

Warburg in the 1920s compared slices of tumour and normal tissues, and found that tumours consume more glucose and produce more lactate, even in the presence of adequate oxygen (Kim and Dang, 2006). These observations were termed the Warburg effect or aerobic glycolysis. Since then, it has been understood that energy metabolism in cancer cells depends on aerobic glycolysis rather than oxidative phosphorylation in mitochondria. The advantages of glycolysis for cancer cells are postulated to be adaptation to hypoxia, acquisition of resistance to mitochondria-mediated apoptosis and acidification of the tumour microenvironment by lactate from cancer cells, which leads to tumour invasion or metastasis (Garber, 2010).

Aerobic glycolysis is regulated by several oncogenes (e.g., AKT, MYC) and HIF1. Myc induces the expression of genes regulating glycolysis, such as GLUT1, HK2, Enolase1 and LDHA (Shim *et al*,

1997; Osthus *et al*, 2000; Dang *et al*, 2009). Under hypoxic conditions, HIF1 also induces the expression of glycolytic enzymes, such as GLUT1, enolase1, LDHA and pyruvate dehydrogenase kinase-1 (PDK1) (Semenza *et al*, 1994; Lu *et al*, 2002; Kim *et al*, 2006). The PDK1 is a Ser/Thr kinase that inactivates mitochondrial pyruvate dehydrogenase (PDH) by phosphorylation. As PDH is a gatekeeper enzyme that converts pyruvate to acetyl-CoA, PDK1 is known as a key regulator of the Warburg effect (Kim and Dang, 2006). Dichloroacetate (DCA), a PDK inhibitor, and 2-deoxyglucose, a hexokinase inhibitor, have been proposed as anticancer agents that target glycolysis (Pelicano *et al*, 2006; Pathania *et al*, 2009).

The LDH is a well-known adverse prognostic factor in multiple myeloma (MM) as a component other than a molecule-regulating glycolysis (Barlogie *et al*, 1989; Dimopoulos *et al*, 1991;

*Correspondence: Associate Professor H Hata;
E-mail: hata@kumamoto-u.ac.jp

Received 18 May 2012; revised 29 October 2012; accepted 30 October 2012;
published online 29 November 2012

© 2013 Cancer Research UK. All rights reserved 0007–0920/13

Anagnostopoulos *et al*, 2005; Terpos *et al*, 2010). However, the mechanism of how LDH contributes to a poor prognosis in MM has not been studied.

In the present study, we investigated whether the Warburg effect is operative in MM cells by analysing the expression of glycolytic enzymes in MM cells, as well as their glucose consumption and lactate production. We also examined whether inhibition of PDK1 can be a therapeutic modality by using reagents that target aerobic glycolysis or inhibit PDK1 mRNA.

MATERIALS AND METHODS

Cell culture. Human myeloma cell lines, KMS-12BM (Ohtsuki *et al*, 1989), KMS-12-PE (Ohtsuki *et al*, 1989), U266 (Ikeyama *et al*, 1986), RPMI8226 (Matsuoka *et al*, 1967), KHM11 (Hata *et al*, 1994), KMM1 (Togawa *et al*, 1982), and a bortezomib-resistant myeloma cell line, KMS11/BTZ (Ri *et al*, 2010), were cultured in RPMI-1640 containing 10% fetal bovine serum at 37 °C under 5% CO₂. The cell concentrations were ascertained by direct counting using Trypan blue.

Patient samples. A total of 59 patients with MM and 13 patients with monoclonal gammopathy of undetermined significance (MGUS) were evaluated in the present study. Clinical specimens were taken from the MM and MGUS patients at the Department of Hematology, Kumamoto University Hospital, under written informed consent. After isolation of mononuclear cells from bone marrow samples using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden), myeloma cells were purified using CD138-immunomagnetic beads (Miltenyi Biotec, Paris, France) as previously described (Uneda *et al*, 2003). Survival data of 54 MM cases were utilised for analysis. Cutoff of LDHA mRNA expression was median value of all cases analysed.

cDNA synthesis and real-time PCR. Using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), RNA was extracted from myeloma cells. The cDNA synthesis was performed using a SuperScript First-Standard Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's protocol. Quantitative PCR analyses were performed using Assay-on-Demand primers and the TaqMan Universal PCR Master Mix reagent (Applied Biosystems, Foster City, NJ, USA). The samples were analysed using an ABI Prism 7700 Sequence Detection System (Applied Biosystems). The $\Delta\Delta CT$ method was utilised to quantify the relative changes in gene expression as described previously (Livak and Schmittgen, 2001). The expression levels of β -actin were used to normalise the relative expression levels. Experiments were duplicated.

Glycolysis inhibitors. Sodium oxamate and DCA were purchased from Sigma-Aldrich (St Louis, MO, USA), and dissolved in water and phosphate-buffered saline, respectively.

Measurement of glucose concentration. The MM cell lines were seeded at 5×10^5 cells ml⁻¹ and incubated for specified periods. A glucose metre (Precision Xceed; Abbot Japan, Tokyo, Japan), which was based on the glucose dehydrogenase method, was utilised to determine the concentrations of glucose in the culture media. Experiments were triplicated and analysed by paired *t*-test.

LDH assay. Intracellular LDH activity was evaluated using cell lysates and an LDH Quantification Kit (BioVision, Milpitas, CA, USA) according to the manufacturer's instructions. Briefly, MM cell lines were cultured at $1-2 \times 10^5$ cells ml⁻¹, and then 2×10^6 cells were harvested and processed for cell lysate preparation. The LDH activities in the cell lysates were determined by monitoring the rate of NADH production from NAD⁺. After incubation of the samples for 30 min at room temperature, the optical densities at a wavelength of 450 nm were analysed using a

spectrophotometer (Vmax kinetic microplate reader; Molecular Devices, Sunnyvale, CA, USA). The data were calculated as the LDH activity per cell lysate protein amount. The protein concentrations in the cell lysates were measured using a BCA Protein Assay Kit (Pierce Biotechnology Inc., Rockford, IL, USA). Experiments were triplicated.

Measurement of lactate production. The MM cell lines were seeded at 5×10^5 cells ml⁻¹ in 12-well plates. The lactate concentrations were evaluated using a lactate metre (Lactate Pro; Arkray, Kyoto, Japan), which electronically analysed potassium ferrocyanide converted from ferricyanide by lactate. The lactate concentrations in the culture media were also assayed using a Lactate Assay Kit (BioVision). Experiments were triplicated.

Western blotting. Cell lysates were prepared using the M-PER mammalian protein extraction reagent (Pierce Biotechnology Inc.) after addition of Halt EDTA-free phosphatase inhibitor cocktail and Halt protease inhibitor cocktail (both from Pierce Biotechnology Inc.). The cell lysates were separated in NuPAGE Bis-Tris precast gels (Invitrogen) and transferred to PVDF membranes using an iBlot Dry Blotting system (Invitrogen). The membranes were blocked with 5% non-fat dry milk dissolved in Tris-buffered saline (TBS) containing 0.5% Tween-20 (TBS-T) for 1 h at room temperature, followed by incubation with the primary antibodies at 4 °C for 18 h. The following primary antibodies were utilised: anti-caspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-PDK1 (Cell Signaling Technology, Beverly, MA, USA) and anti-actin (Santa Cruz Biotechnology). After washing with TBS-T, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, Oxford, UK) diluted in TBS-T for 2 h at room temperature. The antibody-bound proteins were visualised using an ECL plus kit (Amersham Biosciences) and a bio-image analyser (Fuji Film, Tokyo, Japan).

Analysis of apoptosis. The MM cell lines were incubated in the presence of oxamate, DCA or bortezomib for 24 h. Apoptosis in the MM cell lines was quantified by staining with Annexin V-allophycocyanin and propidium iodide (PI). The samples were analysed by flow cytometry (EPICS MCL/XL or FACSCalibur; both from Becton Dickinson, San Jose, CA, USA). In some experiments, apoptosis was analysed after pretreatment with Z-VAD-FMK, a pan-caspase inhibitor, for 30 min. Experiments were triplicated.

Evaluation of reactive oxygen species (ROS). Intracellular ROS production was measured by staining cells with 2',7'-dichlorofluorescein diacetate (Live Cell Fluorescent Reactive Oxygen Species Detection Kit; Marker Gene Technologies Inc., Eugene, OR, USA) and flow cytometry. *tert*-Butylhydroperoxide was evaluated as a positive control. Experiments were duplicated.

RNA interference experiments. An oligonucleotide targeting human PDK1 (5'-CCCGAAGTGAAGTGAAGAA-3'; siPDK1) was purchased from Qiagen (Valencia, CA, USA). Transfections were performed using the HiPerFect Transfection Reagent (Qiagen) according to the manufacturer's protocol. Briefly, 2×10^5 KMM1 cells in 100 μ l of medium were seeded into 24-well plates and transfected with 750 ng of siPDK1 or control siRNA. After designated periods, the transfected cells were simultaneously processed for cell lysate preparation and subjected to western blotting and apoptosis analyses. Quantification of mRNA was analysed by real-time PCR. Experiments were triplicated.

Statistical analysis. Correlations among gene expressions and serum LDH were analysed by Spearman's correlation analysis. The differences in gene expression between MM and MGUS were examined with the Mann-Whitney *U*-test. Values of $P < 0.05$ were considered to indicate statistical significance. Other statistical analyses were performed by *t*-test.

RESULTS

Expression of glycolysis-related enzyme genes in primary myeloma cells. The LDHA mRNA expression was detected at various levels in purified primary myeloma cells (Figure 1A). As shown in Figure 1B, there was a positive correlation between LDHA expression and the serum LDH level ($P<0.01$). Moreover, MM cells with higher LDHA mRNA expression showed poor survival (Figure 1C), indicating that LDHA mRNA expression in myeloma cells may serve as an adverse prognostic indicator, similar to the serum LDH level.

Next, we examined the expression levels of other glycolysis-related enzymes in myeloma cells. The expression levels of LDHA, PDK1 and MYC were significantly higher in myeloma cells from MM patients than in plasma cells from MGUS patients ($P<0.01$, $P<0.001$ and $P<0.05$, respectively; Figure 1D). Except for HIF1 α , the expression levels were significantly correlated with one another (Table 1). These findings indicate that the aerobic glycolysis system is operative in some MM cells.

Analysis of glycolysis in MM cell lines. Consistent with the findings in the primary samples, the glycolysis-related genes LDHA, PDK1 and MYC were expressed at various levels in the MM cell lines (Figure 2A). When we divide cell lines into two groups, 12BM/U266 and others, there was a statistical differences in LDHA and MYC expressions (P value = 0.0027 and 0.0058, respectively), whereas there was no statistical difference in PDK1 expression ($P=0.0817$), although mean expression levels of PDK1 tended to be lower in 12BM/U266 group than that of others (226.2 and 2976.1, respectively).

Similar findings were obtained for LDH activity in the cell lysates of the 8226, 12PE, KHM11 and KMM1 cell lines (Figure 2B). There was a statistical difference in LDH activity of 12BM/U266 group from others ($P<0.0001$). The glucose concentrations in the culture supernatants of these four cell lines (8226, 12PE, KHM11 and KMM1) were dramatically reduced within a few days, whereas the glucose level of the KMS-12BM cell line slowly decreased and that of the U266 cell line was unchanged (Figure 2C). On the other hand, high lactate levels were detected in

the cell lines with high LDH activity (8226, 12PE, KHM11 and KMM1; Figure 2D).

Induction of apoptosis by the LDHA inhibitor oxamate. Oxamate, an inhibitor of LDH that competes with pyruvate for its binding site on the enzyme, induced apoptosis in MM cells. The cells were treated with oxamate at 0, 12.5, 25, 50 or 100 mM for 24 h and then analysed. All the MM cell lines showed apoptosis at variable levels in a dose-dependent manner (Figure 3A). In particular, the cell lines with high LDH activity (12PE, 8226, KHM11 and KMM1) showed higher sensitivity to oxamate than the cell lines with low LDH activity. There was a statistical difference in the amount of apoptotic cells of U266/12BM cells from those of other cell lines induced by oxamate at a concentration of 100 mM. Oxamate induced apoptosis in primary MM cells, but showed no cytotoxicity toward normal peripheral blood mononuclear cells (PBMCs; Figure 3B).

To further characterise the mechanism of the apoptosis induced by oxamate, activation of caspase-3 was analysed. As shown in Figure 3C, cleaved caspase-3 was detected in oxamate-treated 12PE cells. On the other hand, activated caspase-3 was not observed in KMS-12BM cells, which were less sensitive to oxamate. Apoptosis induced by oxamate was partly inhibited by the pan-caspase inhibitor Z-VAD-FMK (Figure 3D).

Table 1. Positive correlations of genes associated with aerobic glycolysis expressed in primary MM cells	
Genes associated with glycolysis	Statistical correlation
LDHA/PDK1	$P=0.0023$ (rs = 0.424)
LDHA/GLUT1	$P=0.0003$ (rs = 0.476)
LDHA/Myc	$P<0.0001$ (rs = 0.74)
GLUT1/PDK1	$P<0.0001$ (rs = 0.54)
GLUT1/Myc	$P<0.0001$ (rs = 0.67)
PDK1/Myc	$P<0.0001$ (rs = 0.503)
Abbreviations: MM = multiple myeloma; LDHA = lactate dehydrogenase A; PDK1 = pyruvate dehydrogenase kinase-1; GLUT1 = glucose transporter 1.

Figure 1. Expression levels of glycolysis-related genes in primary MM cells obtained from 59 cases. (A) Expression levels of LDHA in purified MM cells. The expression levels vary among the cases. (B) LDHA mRNA expression is positively correlated with serum LDH (rs = 0.37, $P<0.01$). (C) Higher expression of LDHA mRNA is associated with poor overall survival ($P<0.01$; median survival durations of the cases with low and high LDH expression were 1549 and 408 days, respectively). $N=54$. (D) Expression levels of glycolytic enzyme genes (LDHA, PDK1 and MYC) in MM and MGUS. The expression levels of all three genes are statistically higher in MM than in MGUS.

Figure 2. Analysis of glycolysis in MM cell lines. (A) Relative gene expression levels (LDHA, PDK1 and MYC) in MM cell lines. The 8226, 12PE, KHM11 and KMM1 cells express LDHA and MYC, whereas 12BM and U266 cells do not ($P=0.0027$ and 0.0058 , respectively). (B) The LDH activities in cell lysates. The LDH activity is lower in 12BM/U266 cells than others ($P<0.0001$). (C) Glucose consumption by MM cell lines. Significant decreases in glucose are observed in the supernatants of 8226, 12PE, KHM11 and KMM1 cells, whereas low consumption of glucose is observed in the supernatants of 12BM and U266 cells. Statistical analyses are shown in a table. *Statistically significant by paired t-test. (D) Lactate production in the culture supernatants of MM cells. The 8226, 12PE, KHM11 and KMM1 cells show more lactate production than 12BM and U266 cells ($P<0.0001$).

Figure 3. Induction of apoptosis in MM cells by oxamate. (A) Oxamate, a competitive inhibitor of LDHA, induces apoptosis in some MM cell lines (8226, 12PE, 8226, KHM11 and KMM1) in a dose-dependent manner, whereas other cell lines (U266 and 12BM) show minimal induction of apoptosis. There was a statistical difference in the amount of apoptotic cells induced by oxamate at a concentration of 100 mM when comparing apoptotic cells in U266/12BM from those in others ($P=0.0002$). (B) Oxamate does not induce apoptosis in PBMCs. (C) Western blot analyses of caspase-3. Oxamate induces caspase-3 in a dose-dependent manner. (D) Inhibition of oxamate-induced apoptosis by the pan-caspase inhibitor ZVAD-FMK. There was a significant inhibition of oxamate-induced apoptosis by ZVAD-FMK.

Figure 4. Dichloroacetate induces apoptosis in MM cells. (A) Induction of Annexin V/PI-positive cells by DCA. The 12PE and KMM1 cells show apoptosis induction by DCA in a dose-dependent manner. *Statistical analysis of Annexin V, **statistical analysis of PI. **(B)** Reactive oxygen species (ROS) production in MM cells treated with DCA. The ROS production is induced by DCA at a concentration of 40 mM. The ROS inducer TBHP was evaluated as a positive control. **(C)** Induction of apoptosis in primary MM cells by DCA (Annexin V/PI staining). **(D)** Lack of cytotoxic effects of DCA toward normal PBMCs (Annexin V/PI staining).

Induction of apoptosis by the PDK1 inhibitor DCA. Next, we investigated whether the PDK inhibitor DCA induced apoptosis in MM cells. The cells were treated with DCA at 0, 10, 20 or 40 mM for 24 h and then subjected to Annexin V/PI analyses. Apoptosis was induced in the 12PE and KMM1 cell lines by DCA in a dose-dependent manner (Figure 4A). Dichloroacetate also induced ROS production at a concentration of 40 mM (Figure 4B). We further found that DCA induced apoptosis in primary MM cells, but showed no cytotoxicity toward normal PBMCs (Figure 4C and D).

We then examined the effects of a combination of DCA and bortezomib, which is a proteasome inhibitor at 4 nM but shows a minimum cytotoxic effect (~20% cell death) at that concentration (Hideshima *et al*, 2001). The combination of DCA (40 mM) with bortezomib (4 nM) showed more induction of apoptosis on KMM1 cells than those when they utilised solely (Figure 5A). To further investigate the mechanism of the cell death induced by the combination of DCA with bortezomib, caspase-3 and ROS production were analysed. A significant increase in cleaved caspase-3 was observed after treatment with the combination of DCA and bortezomib compared with monotherapy (Figure 5B), whereas increase of ROS was marginal by the addition of bortezomib to DCA (Supplementary Figure S1). Interestingly, the bortezomib-resistant myeloma cell line KMS11/BTZ showed significant cell death after DCA treatment compared with the parental bortezomib-sensitive cell line KMS11 (data not shown). Significant induction of apoptosis to KMS11/BTZ was found by the

treatment with oxamate or DCA (Figure 5C and D). Because oxamate solely induced apoptosis to KMS11/BTZ at high levels, augmentation of apoptosis was not found by the combination with bortezomib. Although slight increase of apoptotic cells by bortezomib was found in combination with oxamate at a concentration of 12.5 mM, the amount of increase was not evident. On the other hand, although DCA induced apoptosis of KMS11/BTZ in a dose-dependent manner, augmentation of apoptosis by the addition of bortezomib to DCA was not observed (Figure 5D).

Based on our findings that the PDK1 inhibitor DCA induced apoptosis in MM cells, we investigated the effects of siRNA-mediated inhibition of PDK1 expression. Inhibition of PDK1 expression was confirmed at the mRNA and protein levels at days 1–5 of the treatment (Figure 6A). Significant induction of apoptosis was observed after the PDK1 inhibition (Figure 6B).

DISCUSSION

We found that high LDHA mRNA expression in MM cells was correlated with a high level of serum LDH. As LDH is known to be an adverse prognostic factor in MM (Barlogie *et al*, 1989), our observations are consistent with the higher LDHA expression in MM cells with short survival. Indeed, the serum LDH level was correlated with poor overall survival in our cohort (median: 1559

Figure 5. Cytotoxic effects of DCA in combination with bortezomib. (A) Evaluation of Annexin V/PI staining in KMM1 cells after treatment with DCA (40 nM) alone or in combination with bortezomib (4 nM). Apoptosis was evaluated as fold increase of Annexin V (upper panel) and PI (lower panel) compared with control. Significant augmentation of apoptotic cells was found when DCA and bortezomib were combined. (B) Western blot analyses of caspase-3. The combination of bortezomib (bor) and DCA causes increased activation of caspase-3 compared with monotherapy in a MM cell line, KMM-1. (C,D) Induction of apoptosis by combination of oxamate (C) or DCA (D) with bortezomib. Open bars: control; grey bars: oxamate or DCA monotherapy; solid bars: bortezomib alone or in combination with oxamate or DCA. There is no increase of oxamate/DCA-induced apoptosis by bortezomib despite slight increase of apoptosis was found in the combination of bortezomib with oxamate at 12.5 nM.

Figure 6. Inhibition of PDK1 expression induces apoptosis in MM cells. (A) Confirmation of siRNA-mediated inhibition of PDK1 expression in KMM1 cells by real-time PCR (upper panel) and western blotting (lower panel). (B) Inhibition of PDK1 expression results in apoptosis induction in KMM1 cells (Annexin V/PI staining). Apoptosis was analysed at 120 h from the addition of siRNA. Upper panel: representative results of quadruplicated experiments. Lower panel: mean value of Annexin V and PI staining. Knockdown of PDK1 significantly increased apoptosis.

vs 331 days, $P=0.01$). We evaluated other molecules, PDK1, cMYC, GLUT1 and HIF1A, if these serve as prognostic factor. However, in our cohort, LDHA expression was the only significant prognostic factor (Supplementary Figure S2). Similar finding was obtained by the analysis of microarray database referring total therapy 2 (TT2; NCBI data set no. GSE2658; Zhan *et al*, 2006), although difference of survival was marginal, possibly because of high intensity of chemotherapy (Supplementary Figure S3). We also made analysis of subgroup of TT2 cases, which was classified by gene expression profiles and found that CD2 group showed lower expression of LDHA (Supplementary Figure S4) (Zhan *et al*, 2006). As CD2 group showed longest overall survival, low expression of LDHA may be a marker of low-risk MM. Analysis of these gene expression in human myeloma cell lines, which were classified in six subgroups by expression of MAF, MMSET, CCND1, FRZB with or without overexpression of cancer testis antigens (Moreaux *et al*, 2011), showed no specific expression pattern of these genes within molecular subgroups (Supplementary Figure S5). This may reflect somewhat uniform background of cell lines than those of primary samples as most cell lines were established from extra medullary lesions with aggressive disease status. As LDH is a glycolytic enzyme that converts pyruvate to lactate, MM cells with high LDHA expression may have high glycolytic activity. Indeed, LDHA expression was correlated with the expression levels of other glycolytic enzymes. Moreover, glucose consumption and lactate production were correlated with the expression levels of these genes. This is the first report showing the significance of LDH as a glycolytic enzyme in MM cells other than only as an adverse prognostic factor.

The mechanisms that regulate glycolysis represent an interesting issue. It is considered that myeloma cells accumulate various abnormalities that contribute to disease progression from MGUS (Korde *et al*, 2011; Zingone and Kuehl, 2011). We found that LDHA expression was significantly higher in MM cells than in plasma cells from patients with MGUS. Similar to the findings for LDHA expression, we found that MYC expression was significantly higher in MM cells than in plasma cells from patients with MGUS, as previously reported (Shou *et al*, 2000; Gabrea *et al*, 2006; Chng *et al*, 2011). It has been reported that MYC is activated in 67% of MM patients, but is not or rarely expressed in MGUS (Chng *et al*, 2011). Shou *et al* (2000) showed that dysregulation of c-myc is principally caused by complex genomic rearrangements that occur during the late stages of MM. Thus, it is thought that MYC gene expression occurs as a late event during the progression of MM. Furthermore, MYC was reported to be a direct target of the transcription factor interferon regulatory factor-4 (IRF4), which is known to be an important oncogene in the pathogenesis of MM (Shaffer *et al*, 2008, 2009). The same authors further reported that genes associated with glycolysis (LDHA, HK and PDK1) are also IRF4-targeted genes (Shaffer *et al*, 2008, 2009). These previous studies support our findings that aerobic glycolysis is upregulated in MM cells compared with MGUS cells, possibly through MYC activation.

With regard to novel therapeutic approaches toward MM cells in cases with a poor prognosis, targeting of the glycolytic pathway should be reasonable. A small-molecule inhibitor of LDHA, FX11, was reported to trigger oxidative stress in cancer cells, leading to necrotic cell death (Le *et al*, 2010). The same authors further found that a reduction in the LDHA activity was associated with an elevation of the NADH/NAD⁺ ratio, which was linked to increased ROS production and cell death. Moreover, it was reported that LDHA inhibition leads to a reduction in lactate production, which is the energy source for cancer cells, and induces cell death (Xie *et al*, 2009; Le *et al*, 2010). In another study, a reduction in LDHA activity was found to cause a decrease in the mitochondrial membrane potential (Fantin *et al*, 2006). We found that oxamate, a competitive inhibitor of the LDH enzyme, induced

apoptosis by activating caspase-3, especially in MM cells with high LDH activity. Taken together, the present findings and those in previous reports suggest that inhibition of glycolysis can be a new therapeutic modality for MM cells with high LDH expression.

Dichloroacetate, a PDK inhibitor that binds to the N-terminal domain of PDK2, also decreases PDK1 activity (Kato *et al*, 2007). It has been shown to possess anticancer activities by inducing cell cycle arrest and depolarising the hyperpolarized inner mitochondrial membrane potential (Michelakis *et al*, 2008; Wong *et al*, 2008; Madhok *et al*, 2010; Sun *et al*, 2010, 2011; Tong *et al*, 2011). Oral DCA was reported to show good bioavailability, which encourages phase I/II clinical trials for its use in brain cancer and non-small lung cancer patients (Michelakis *et al*, 2010; Porporato *et al*, 2011). A new selective PDK1 inhibitor, AZD7545, is already expected to undergo a clinical trial (Kato *et al*, 2007). As PDK1 shows relatively higher expression in plasma cells or myeloma cells compared with other haematopoietic lineages (Shaffer *et al*, 2008; Jourdan *et al*, 2009), targeting of PDK1 should be more suitable than targeting LDH, which basically exists in all kinds of cells. Indeed, PDK1 was reported to be expressed at only low levels in most normal tissues (Jourdan *et al*, 2009), as can be seen in the open web ATLAS (<http://amazonia.transcriptome.eu/>) (Carrouer *et al*, 2010).

We have found for the first time, to our knowledge, that DCA induced ROS production and apoptosis in MM cell lines and primary MM cells. Moreover, we found that inhibition of PDK1 mRNA expression induced ROS production, activated caspase-3 and led to apoptosis in MM cells. These findings suggest that PDK1 could also be a therapeutic target molecule for multiple myeloma.

Bortezomib is a well-known proteasome inhibitor with significant anti-myeloma activity through inhibition of NF- κ B activity, cytokine secretion and angiogenesis (Hideshima *et al*, 2009; Hideshima *et al*, 2011). Although bortezomib shows high response rates, resistance to bortezomib remains a problem. Consistent with reports that combination therapies of glycolytic inhibitors with anticancer drugs show synergistic or additive effects (Zhou *et al*, 2010; Fiume *et al*, 2011; Tong *et al*, 2011), we found an additive effect of DCA and bortezomib, which was accompanied by induction of caspase-3 activation and ROS production. Dichloroacetate induced apoptosis in bortezomib-resistant cells in a dose-dependent manner. However, as we did not find a significant additive effect of DCA and bortezomib to bortezomib-resistant cells, PDK1 inhibitor alone may not sensitise bortezomib-resistant cells to bortezomib.

Taken together, the present data suggest that targeting glycolysis, especially PDK1, could be a new strategy for MM patients with high serum LDH levels and a poor prognosis.

ACKNOWLEDGEMENTS

A bortezomib-resistant myeloma cell line, KMS11/BTZ, was kindly provided by Dr Masaki Ri and Dr Shunsuke Iida (Department of Medical Oncology and Immunology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Aichi, Japan), and by Dr Yukimasa Shiotsu and Dr Takayuki Nakashima (Research Division, Kyowa Hakko Kirin Co. Ltd, Tokyo, Japan). We thank Dr Eisaku Iwanaga (Department of Hematology, Kumamoto University Hospital, Kumamoto, Japan) for helpful support for microarray database analysis.

REFERENCES

- Anagnostopoulos A, Gika D, Symeonidis A, Zervas K, Pouli A, Repoussis P, Grigoraki V, Anagnostopoulos N, Economopoulos T, Maniatis A,

- Dimopoulos MA (2005) Multiple myeloma in elderly patients: prognostic factors and outcome. *Eur J Haematol* **75**: 370–375.
- Barlogie B, Smallwood L, Smith T, Alexanian R (1989) High serum levels of lactic dehydrogenase identify a high-grade lymphoma-like myeloma. *Ann Intern Med* **110**: 521–525.
- Carrouer TL, Assou S, Tondeur S, Lhermitte L, Lamb N, Reme T, Pantesco V, Hamamah S, Klein B, De Vos J (2010) Amazonia!: an online resource to google and visualize public human whole genome expression data. *Open Bioinformatics J* **4**: 5–10.
- Chng WJ, Huang GF, Chung TH, Ng SB, Gonzalez-Paz N, Troska-Price T, Mulligan G, Chesi M, Bergsagel PL, Fonseca R (2011) Clinical and biological implications of MYC activation: a common difference between MGUS and newly diagnosed multiple myeloma. *Leukemia* **25**: 1026–1035.
- Dang CV, Le A, Gao P (2009) MYC-induced cancer cell energy metabolism and therapeutic opportunities. *Clin Cancer Res* **15**: 6479–6483.
- Dimopoulos MA, Barlogie B, Smith TL, Alexanian R (1991) High serum lactate dehydrogenase level as a marker for drug resistance and short survival in multiple myeloma. *Ann Intern Med* **115**: 931–935.
- Fantin VR, St-Pierre J, Leder P (2006) Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology, and tumor maintenance. *Cancer Cell* **9**: 425–434.
- Fiume L, Vettriano M, Manerba M, Di Stefano G (2011) Inhibition of lactic dehydrogenase as a way to increase the anti-proliferative effect of multi-targeted kinase inhibitors. *Pharmacol Res* **63**: 328–334.
- Gabrea A, Leif Bergsagel P, Michael Kuehl W (2006) Distinguishing primary and secondary translocations in multiple myeloma. *DNA Repair (Amst)* **5**: 1225–1233.
- Garber K (2010) Oncology's energetic pipeline. *Nat Biotechnol* **28**: 888–891.
- Hata H, Matsuzaki H, Sonoki T, Takemoto S, Kuribayashi N, Nagasaki A, Takatsuki K (1994) Establishment of a CD45-positive immature plasma cell line from an aggressive multiple myeloma with high serum lactate dehydrogenase. *Leukemia* **8**: 1768–1773.
- Hideshima T, Ikeda H, Chauhan D, Okawa Y, Raje N, Podar K, Mitsiades C, Munshi NC, Richardson PG, Carrasco RD, Anderson KC (2009) Bortezomib induces canonical nuclear factor-kappaB activation in multiple myeloma cells. *Blood* **114**: 1046–1052.
- Hideshima T, Richardson P, Chauhan D, Palombella VJ, Elliott PJ, Adams J, Anderson KC (2001) The proteasome inhibitor PS-341 inhibits growth, induces apoptosis, and overcomes drug resistance in human multiple myeloma cells. *Cancer Res* **61**: 3071–3076.
- Hideshima T, Richardson PG, Anderson KC (2011) Mechanism of action of proteasome inhibitors and deacetylase inhibitors and the biological basis of synergy in multiple myeloma. *Mol Cancer Ther* **10**: 2034–2042.
- Ikeyama S, Nakagawa S, Arakawa M, Sugino H, Kakinuma A (1986) Purification and characterization of IgE produced by human myeloma cell line, U266. *Mol Immunol* **23**: 159–167.
- Jourdan M, Caraux A, De Vos J, Fiol G, Larroque M, Cognot C, Bret C, Duperray C, Hose D, Klein B (2009) An in vitro model of differentiation of memory B cells into plasmablasts and plasma cells including detailed phenotypic and molecular characterization. *Blood* **114**: 5173–5181.
- Kato M, Li J, Chuang JL, Chuang DT (2007) Distinct structural mechanisms for inhibition of pyruvate dehydrogenase kinase isoforms by AZD7545, dichloroacetate, and radicicol. *Structure* **15**: 992–1004.
- Kim JW, Dang CV (2006) Cancer's molecular sweet tooth and the Warburg effect. *Cancer Res* **66**: 8927–8930.
- Kim JW, Tchernyshyov I, Semenza GL, Dang CV (2006) HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. *Cell Metab* **3**: 177–185.
- Korde N, Kristinsson SY, Landgren O (2011) Monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma (SMM): novel biological insights and development of early treatment strategies. *Blood* **117**: 5573–5581.
- Le A, Cooper CR, Gouw AM, Dinavahi R, Maitra A, Deck LM, Royer RE, Vander Jagt DL, Semenza GL, Dang CV (2010) Inhibition of lactate dehydrogenase A induces oxidative stress and inhibits tumor progression. *Proc Natl Acad Sci USA* **107**: 2037–2042.
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**: 402–408.
- Lu H, Forbes RA, Verma A (2002) Hypoxia-inducible factor 1 activation by aerobic glycolysis implicates the Warburg effect in carcinogenesis. *J Biol Chem* **277**: 23111–23115.
- Madhok BM, Yeluri S, Perry SL, Hughes TA, Jayne DG (2010) Dichloroacetate induces apoptosis and cell-cycle arrest in colorectal cancer cells. *Br J Cancer* **102**: 1746–1752.
- Matsuoka Y, Moore GE, Yagi Y, Pressman D (1967) Production of free light chains of immunoglobulin by a hematopoietic cell line derived from a patient with multiple myeloma. *Proc Soc Exp Biol Med* **125**: 1246–1250.
- Michelakis ED, Sutendra G, Dromparis P, Webster L, Haromy A, Niven E, Maguire C, Gammer TL, Mackey JR, Fulton D, Abdulkarim B, McMurtry MS, Petruk KC (2010) Metabolic modulation of glioblastoma with dichloroacetate. *Sci Transl Med* **2**: 31ra34.
- Michelakis ED, Webster L, Mackey JR (2008) Dichloroacetate (DCA) as a potential metabolic-targeting therapy for cancer. *Br J Cancer* **99**: 989–994.
- Moreaux J, Klein B, Bataille R, Descamps G, Maiga S, Hose D, Goldschmidt H, Jauch A, Reme T, Jourdan M, Amiot M, Pellat-Deceunynck C (2011) A high-risk signature for patients with multiple myeloma established from the molecular classification of human myeloma cell lines. *Haematologica* **96**: 574–582.
- Ohtsuki T, Yawata Y, Wada H, Sugihara T, Mori M, Namba M (1989) Two human myeloma cell lines, amylase-producing KMS-12-PE and amylase-non-producing KMS-12-BM, were established from a patient, having the same chromosome marker, t(11;14)(q13;q32). *Br J Haematol* **73**: 199–204.
- Osthus RC, Shim H, Kim S, Li Q, Reddy R, Mukherjee M, Xu Y, Wonsey D, Lee LA, Dang CV (2000) Deregulation of glucose transporter 1 and glycolytic gene expression by c-Myc. *J Biol Chem* **275**: 21797–21800.
- Pathania D, Millard M, Neamati N (2009) Opportunities in discovery and delivery of anticancer drugs targeting mitochondria and cancer cell metabolism. *Adv Drug Deliv Rev* **61**: 1250–1275.
- Pelicano H, Martin DS, Xu RH, Huang P (2006) Glycolysis inhibition for anticancer treatment. *Oncogene* **25**: 4633–4646.
- Porporato PE, Dhup S, Dadhich RK, Copetti T, Sonveaux P (2011) Anticancer targets in the glycolytic metabolism of tumors: a comprehensive review. *Front Pharmacol* **2**: 49.
- Ri M, Iida S, Nakashima T, Miyazaki H, Mori F, Ito A, Inagaki A, Kusumoto S, Ishida T, Komatsu H, Shiotsu Y, Ueda R (2010) Bortezomib-resistant myeloma cell lines: a role for mutated PSMB5 in preventing the accumulation of unfolded proteins and fatal ER stress. *Leukemia* **24**: 1506–1512.
- Semenza GL, Roth PH, Fang HM, Wang GL (1994) Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. *J Biol Chem* **269**: 23757–23763.
- Shaffer AL, Emre NC, Lamy L, Ngo VN, Wright G, Xiao W, Powell J, Dave S, Yu X, Zhao H, Zeng Y, Chen B, Epstein J, Staudt LM (2008) IRF4 addiction in multiple myeloma. *Nature* **454**: 226–231.
- Shaffer AL, Emre NC, Romesser PB, Staudt LM (2009) IRF4: Immunity. Malignancy! Therapy? *Clin Cancer Res* **15**: 2954–2961.
- Shim H, Dolde C, Lewis BC, Wu CS, Dang G, Jungmann RA, Dalla-Favera R, Dang CV (1997) c-Myc transactivation of LDH-A: implications for tumor metabolism and growth. *Proc Natl Acad Sci USA* **94**: 6658–6663.
- Shou Y, Martelli ML, Gabrea A, Qi Y, Brents LA, Roschke A, Dewald G, Kirsch IR, Bergsagel PL, Kuehl WM (2000) Diverse karyotypic abnormalities of the c-myc locus associated with c-myc dysregulation and tumor progression in multiple myeloma. *Proc Natl Acad Sci USA* **97**: 228–233.
- Sun RC, Board PG, Blackburn AC (2011) Targeting metabolism with arsenic trioxide and dichloroacetate in breast cancer cells. *Mol Cancer* **10**: 142.
- Sun RC, Fadia M, Dahlstrom JE, Parish CR, Board PG, Blackburn AC (2010) Reversal of the glycolytic phenotype by dichloroacetate inhibits metastatic breast cancer cell growth in vitro and in vivo. *Breast Cancer Res Treat* **120**: 253–260.
- Terpos E, Katodritou E, Roussou M, Pouli A, Michalis E, Delimpasi S, Pargharidou A, Kartasis Z, Zomas A, Symeonidis A, Viniou NA, Anagnostopoulos N, Economopoulos T, Zervas K, Dimopoulos MA (2010) High serum lactate dehydrogenase adds prognostic value to the international myeloma staging system even in the era of novel agents. *Eur J Haematol* **85**: 114–119.
- Togawa A, Inoue N, Miyamoto K, Hyodo H, Namba M (1982) Establishment and characterization of a human myeloma cell line (KMM-1). *Int J Cancer* **29**: 495–500.
- Tong J, Xie G, He J, Li J, Pan F, Liang H (2011) Synergistic antitumor effect of dichloroacetate in combination with 5-fluorouracil in colorectal cancer. *J Biomed Biotechnol* **2011**: 740564.
- Uneda S, Hata H, Matsuno F, Harada N, Mitsuya Y, Kawano F, Mitsuya H (2003) Macrophage inflammatory protein-1 alpha is produced by human

- multiple myeloma (MM) cells and its expression correlates with bone lesions in patients with MM. *Br J Haematol* **120**: 53–55.
- Wong JY, Huggins GS, Debidia M, Munshi NC, De Vivo I (2008) Dichloroacetate induces apoptosis in endometrial cancer cells. *Gynecol Oncol* **109**: 394–402.
- Xie H, Valera VA, Merino MJ, Amato AM, Signoretti S, Linehan WM, Sukhatme VP, Seth P (2009) LDH-A inhibition, a therapeutic strategy for treatment of hereditary leiomyomatosis and renal cell cancer. *Mol Cancer Ther* **8**: 626–635.
- Zhan F, Huang Y, Colla S, Stewart JP, Hanamura I, Gupta S, Epstein J, Yaccoby S, Sawyer J, Burington B, Anaissie E, Hollmig K, Pineda-Roman M, Tricot G, van Rhee F, Walker R, Zangari M, Crowley J, Barlogie B, Shaughnessy Jr JD (2006) The molecular classification of multiple myeloma. *Blood* **108**: 2020–2028.
- Zhou M, Zhao Y, Ding Y, Liu H, Liu Z, Fodstad O, Riker AI, Kamarajugadda S, Lu J, Owen LB, Ledoux SP, Tan M (2010) Warburg effect in chemosensitivity: targeting lactate dehydrogenase-A re-sensitizes taxol-resistant cancer cells to taxol. *Mol Cancer* **9**: 33.
- Zingone A, Kuehl WM (2011) Pathogenesis of monoclonal gammopathy of undetermined significance and progression to multiple myeloma. *Semin Hematol* **48**: 4–12.

This work is licensed under the Creative Commons Attribution-NonCommercial-Share Alike 3.0 Unported License. To view a copy of this license, visit <http://creativecommons.org/licenses/by-nc-sa/3.0/>

Supplementary Information accompanies this paper on British Journal of Cancer website (<http://www.nature.com/bjc>)