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ORIGINAL ARTICLE

Promotion of glioma cell survival by acyl-CoA synthetase 5 under extracellular acidosis conditions

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Extracellular acidosis (low pH) is a tumor microenvironmental stressor that has a critical function in the malignant progression and metastatic dissemination of tumors. To survive under stress conditions, tumor cells must evolve resistance to stress-induced toxicity. Acyl-CoA synthetase 5 (ACSL5) is a member of the ACS family, which converts fatty acid to acyl-CoA. ACSL5 is frequently overexpressed in malignant glioma, whereas its functional significance is still unknown. Using retrovirus-mediated stable gene transfer (gain of function) and small interfering RNA-mediated gene silencing (loss of function), we show here that ACSL5 selectively promotes human glioma cell survival under extracellular acidosis. ACSL5 enhanced cell survival through its ACS catalytic activity. To clarify the genome-wide changes in cell signaling pathways by ACSL5, we performed cDNA microarray analysis and identified an ACSL5-dependent gene expression signature. The analysis revealed that ACSL5 was critical to the expression of tumor-related factors including midkine (MDK), a heparin-binding growth factor frequently overexpressed in cancer. Knockdown of MDK expression significantly attenuated ACSL5-mediated survival under acidic state. These results indicate that ACSL5 is a critical factor for survival of glioma cells under acidic tumor microenvironment, thus providing novel molecular basis for cancer therapy.
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Introduction

Enhanced lipid biosynthesis occurs selectively in tumor cells and is closely linked with tumorigenesis (Menendez and Lupu, 2007). In tumor cells, the supply of cellular fatty acid is highly dependent on *de novo* synthesis, and several enzymes in the lipid biosynthesis pathways are involved in tumor cell survival (Brusselmans *et al.*, 2005; Hatzivassiliou *et al.*, 2005; Kuhajda, 2006). These observations suggest that mediators of lipid metabolism are newly recognized molecular targets to induce selective tumor cell death.

Acyl-CoA synthetases (ACSs) are enzymes that convert long-chain fatty acids to acyl-CoA. This reaction is a critical step in several lipid metabolic pathways, including phospholipid biosynthesis, lipid modification of cellular proteins and β-oxidation (Coleman *et al.*, 2002). ACSs are overexpressed in a variety of cancers (Cao *et al.*, 2000, 2001; Yamashita *et al.*, 2000; Sung *et al.*, 2003, 2007; Gassler *et al.*, 2005; Liang *et al.*, 2005; Yeh *et al.*, 2006). Moreover, our recent screening identified an ACS inhibitor as a tumor-selective inducer of apoptosis (Mashima *et al.*, 2005; Mashima and Tsuruo, 2005). These data suggest that ACSs are predominantly involved in tumor cell survival.

Acyl-CoA synthetase 5 (ACSL5) is a unique isozyme among the ACS members, as it is the only known ACS isozyme that localizes on mitochondria (Lewin *et al.*, 2001; Coleman *et al.*, 2002). In human glioma, aberrations occur on chromosome 10q25.1–q25.2, on which the ACSL5 gene is located, and ACSL5 is frequently overexpressed (Yamashita *et al.*, 2000). These observations strongly suggest potential functions of the enzyme in the growth or malignancy of glioma. At present, however, the precise functions of ACSL5 in cancer have not been elucidated.

Extracellular acidosis (low pH) is a tumor microenvironmental stressor (Vaupel *et al.*, 1989). Solid tumors are commonly characterized by a unique pathophysiologic microenvironment (Tannock and Rotin, 1989; Vaupel *et al.*, 1989; Tomida and Tsuruo, 1999). This hostile microenvironment activates several intracellular signaling pathways that promote malignant progression and metastatic dissemination (Harris, 2002;

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Rofstad et al., 2006; van den Beucken et al., 2006). On the other hand, to survive under such stress conditions, tumor cells must also develop resistance to the micro-environmental stress-induced cytotoxicity (Graeber et al., 1996), although the underlying mechanisms remain unclear.

Midkine (MDK) is a basic heparin-binding growth factor of low molecular weight, a member of the neurite growth-promoting factor family (Kadomatsu and Muramatsu, 2004). MDK shows highly increased expression in a number of malignant tumors (Nakagawara et al., 1995; O'Brien et al., 1996; Mishima et al., 1997; Ye et al., 1999; Ikematsu et al., 2000; Jia et al., 2007; Maeda et al., 2007) and enhances tumor progression by promoting survival, growth, migration and angiogenic activity (Kadomatsu et al., 1997; Takei et al., 2001; Kadomatsu and Muramatsu, 2004; Mirkin et al., 2005; Tong et al., 2007). In human brain tumors, especially MDK is overexpressed during tumor progression, and patients whose tumors express a higher level of MDK have a worse prognosis (Mishima et al., 1997).

In this study, we examined the function of ACSL5 in glioma cell survival under extracellular acidosis conditions. Moreover, the ACSL5-regulated gene signature was analysed. The analysis revealed that ACSL5 is a critical regulator of tumor-related genes including MDK.

Results

ACSL5 promotes human glioma cell survival under extracellular acidosis conditions

To clarify the function of ACSL5 in glioma cell survival, we examined the effect of its overexpression on cell survival under various tumor-related stress conditions. We initially examined the expression of endogenous ACSL5 in human glioma cell lines. As a result, we found two cell lines with low levels of ACSL5, SF268 and U251, and two cell lines with relatively high amounts of ACSL5, SNB78 and A1207 (data not shown; see Figure 2a). We stably transduced SF268 cells with a retroviral vector harboring a human ACSL5 gene with a FLAG tag at its carboxy end. Overexpression of FLAG-tagged ACSL5 in the transduced cells (SF268/ACSL5) was confirmed by immunoblot analysis (Figure 1a). Under normal culture conditions, both SF268/mock and SF268/ACSL5 cells showed similar growth rates (Supplementary Figure 1a). By contrast, SF268/ACSL5 showed markedly enhanced survival under extracellular acidosis conditions (pH 6.5) (Figures 1b and c). Similar results were obtained in another human glioma cell line, U251, when it was stably transduced with ACSL5 (data not shown). The major source of proton ion *in vivo* is lactic acid. Therefore, we also examined cell survival under low pH conditions (pH 6.3–6.5) that were generated by lactic acid. As a result, we found that ACSL5 expression also promoted cell survival under lactic acid-based low pH conditions (Supplementary Figure 1b). Extracellular acidosis (range pH 5.8–7.6) is known as one of the pathophysiologic microenvironmental stresses that are characteristically observed in solid tumors (Tannock and Rotin, 1989; Vaupel et al., 1989; Tomida and Tsuruo, 1999). ACSL5-mediated promotion of survival was selective under acidosis conditions, as SF268/ACSL5 did not show apparent survival advantage under other tumor-related stresses such as hypoxia and low serum conditions (Figure 1d).

We have shown earlier that inhibition of total cellular ACS induces cell death through the activation of caspases, the cysteine proteases that have a central function in apoptosis induction (Mashima et al., 2005). To characterize the molecular mechanisms of the reduced cell viability under low pH, we next examined the involvement of a caspase-mediated pathway. As shown in Supplementary Figure 2a, treatment with a specific caspase inhibitor, Z-VAD-fmk, did not recover the reduced SF268 cell viability under low pH. Consistently, caspase protease activity was not elevated in the cells exposed to extracellular acidosis and neither was it affected by ACSL5 expression (Supplementary Figure 2b). Flow cytometric analysis further revealed that the loss of viability under low pH did not accompany the emergence of the sub-G1 population, a characteristic of apoptotic cells (Supplementary Figure 2c). These results indicate that the reduced cell viability under acidosis is caspase-independent and non-apoptotic.

To confirm the function of ACSL5 under acidic conditions, a loss-of-function study was performed using the small interfering RNA (siRNA) against endogenous ACSL5. We found two ACSL5-overexpressed glioma cell lines, SNB78 and A1207 (Figure 2a), and used these cell lines for the loss-of-function study. When SNB78 cells were transfected with ACSL5-siRNAs (si1 and si2), the level of ACSL5 mRNA was clearly reduced in the ACSL5 siRNA-transfected cells (Supplementary Figure 3a). Consistently, the ACSL5 protein was decreased in the SNB78 cells treated with ACSL5 siRNAs (Figure 2b). We found that the inhibition of ACSL5 expression significantly reduced cell viability under the acidic state (pH 6.5) (Figure 2c, right), whereas it did not influence cell survival under normal conditions (pH 7.3) in SNB78 cells (Figure 2c, left and Supplementary Figure 3b). We observed similar results in A1207 cells (Figures 2b and d), except for slight suppression of A1207 cell growth under normal conditions (pH7.3) by one of the ACSL5 siRNAs (siRNA 1). The growth inhibition by siRNA1 could result from its off-target effect, as the other ACSL5 siRNA (siRNA 2) did not show any growth inhibitory effect under normal conditions. By contrast, the inhibition of ACSL5 expression did not reduce cell viability under low serum conditions (Supplementary Figure 3c). To clarify the function of overexpressed ACSL5 in *in vivo* growth of tumor, we further tested the effect of ACSL5 siRNA treatment on ACSL5-overexpressed tumor. For this study, we chose human glioma A1207 cells, as they overexpress endogenous ACSL5 and are tumorigenic in nude mice (Mishima et al., 2001). As a result, we found that *in vivo* treatment

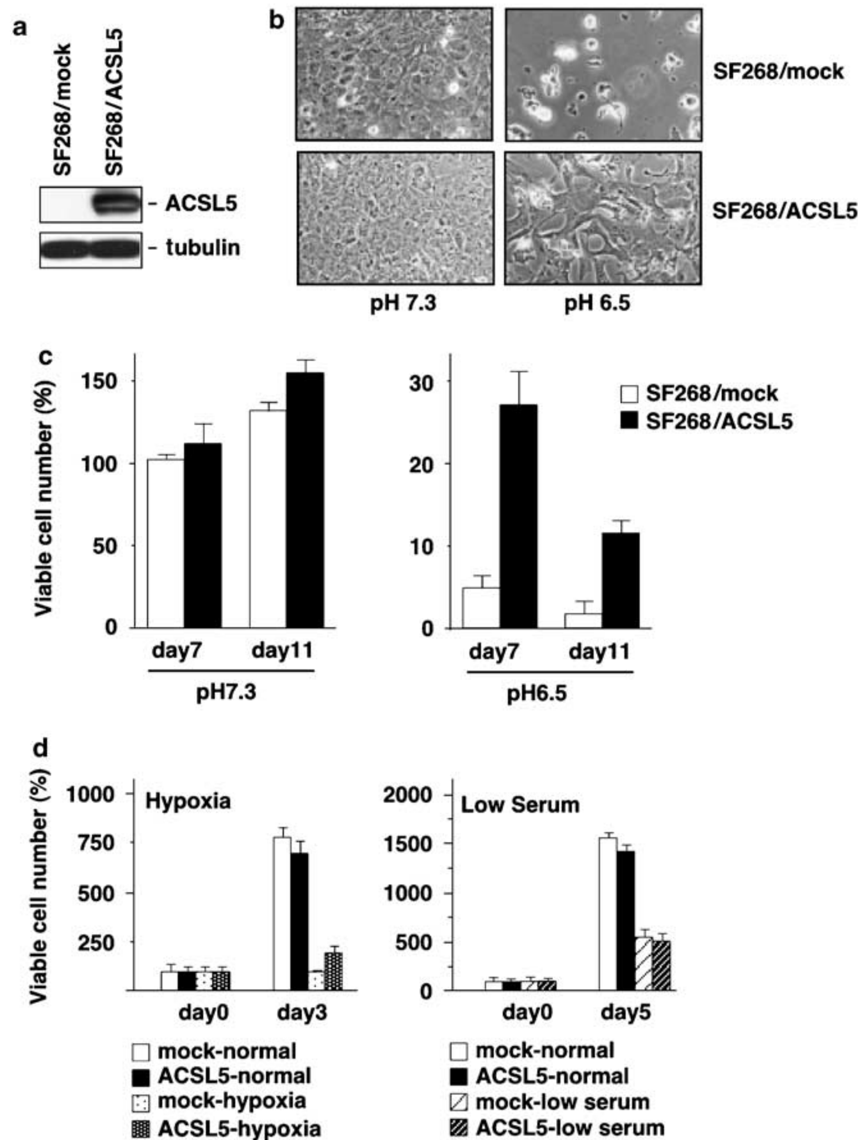

Figure 1 Acyl-CoA synthetase 5 (ACSL5) promotion of survival of human glioma SF268 cells under extracellular acidosis conditions. (**a**) The expression of FLAG epitope-tagged ACSL5 in transduced SF268 cells as revealed by western blot analysis with an anti-ACSL5 antibody. The expressions of α-tubulin were measured as loading controls. (**b** and **c**) Cells were initially seeded on day 0 and maintained under normal (pH 7.3) or acidic (pH 6.5) conditions. Morphologies of the cells on day 7 are shown in (**b**). Cell numbers were counted on days 7 and 11 (**c**). Data are mean values of three independent experiments, and error bars show standard deviations. (**d**) Cells were initially seeded on day 0 and maintained at normal pH levels under hypoxic or low serum (0.1% fetal bovine serum (FBS)) culture conditions. Cell numbers were counted on days 0 and 3 (for hypoxia treatment) or on days 0 and 5 (for low serum treatment). Data are mean values of three independent experiments, and error bars show standard deviations.

with the ACSL5 siRNAs significantly suppressed the growth of A1207 tumor (Supplementary Figure 3d). These results indicate that ACSL5 selectively promotes glioma cell survival under extracellular acidosis and could have a function in tumor survival *in vivo*.

ACSL5 catalytic activity-dependent cell survival under extracellular acidosis conditions

To test whether ACS catalytic activity is required for ACSL5-mediated promotion of survival under acidosis, we constructed an inactive mutant of ACSL5 (ACSL5-MT) (Figure 3a; see Materials and methods). When retrovirally transduced in SF268 cells, the ACSL5-MT protein was expressed stably at a similar level as wild-type ACSL5 (Figure 3b). On the other hand, ACS activity was exclusively elevated in ACSL5-expressed cells but not in ACSL5-MT-expressed cells (Figure 3c), indicating that the ACSL5-MT is actually an inactive mutant. We compared cell survival of these cells under normal and low pH conditions. As shown in Figure 3d, the ACSL5-MT-expressed cells had no survival advantage under acidosis conditions, whereas the wild-type ACSL5-expressed cells did so. These results indicate that

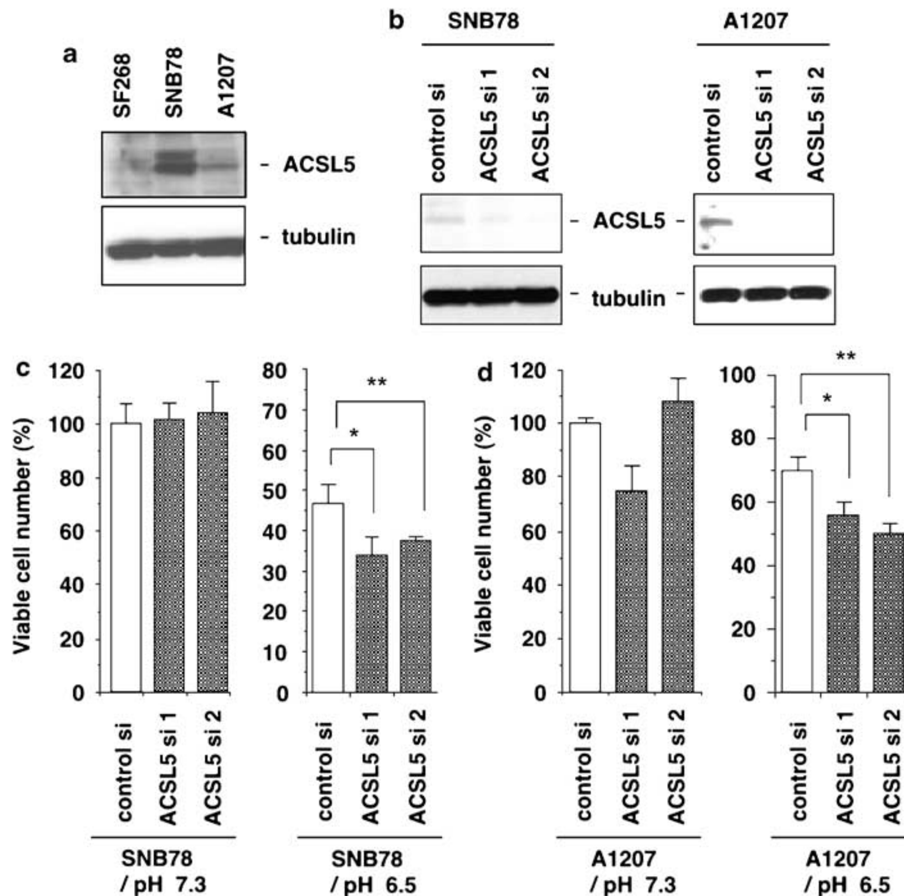

Figure 2 Involvement of endogenously overexpressed acyl-CoA synthetase 5 (ACSL5) in survival of human glioma SNB78 cells under extracellular acidosis conditions. (**a**) Protein expression of endogenous ACSL5 in human glioma cell lines as revealed by western blot analysis with an anti-ACSL5 antibody. The expressions of α-tubulin were measured as loading controls. (**b**) Protein expression of ACSL5 in cells treated with siRNAs. SNB78 and A1207 cells were treated with ACSL5 siRNAs or control siRNA and cultured for 48 h under acidic conditions (pH 6.5). Cell lysates were then prepared, and the expressions of endogenous ACSL5 were detected by an anti-ACSL5 antibody. (**c** and **d**) Viability of SNB78 and A1207 cells after ACSL5 knockdown under normal and acidic conditions. SNB78 and A1207 cells treated with ACSL5-targeted siRNAs or with control siRNA were cultured under normal (pH 7.3) or low pH (pH 6.5) conditions for 4 and 6 days, respectively. Viable cell numbers were counted. Data are mean values of three independent experiments, and error bars show standard deviations. *P*-values (two-sided) were calculated using the Student's *t*-test. *P*-values of <0.05 were considered statistically significant. $^{**}P<0.01$; $^{*}P<0.05$.

ACSL5 promotes survival under low pH conditions through its ACS catalytic activity.

A previous report has shown that ACSL5 selectively promotes the uptake of extracellular palmitic acid. Moreover, palmitic acid enhances the growth of U87MG human glioma cells overexpressed with ACSL5 (Yamashita *et al*., 2000). Therefore, we examined the involvement of extracellular palmitic acid on cell survival under acidosis. However, palmitic acid treatment did not affect cell viability under acidic conditions in SF268 cells (Supplementary Figure 4a). This result indicates that extracellular palmitic acid is not involved in cell survival under low pH.

ACSL5 localizes on mitochondria and is thought to be involved in β-oxidation of fatty acids (Coleman *et al*., 2002). As the β-oxidation pathway leads to a cellular energy supply through ATP production, we speculated that the supply of ATP through ACSL5-mediated β-oxidation could be critical for survival promotion under acidic stress. To test this hypothesis, we examined the change in the cellular ATP level after exposure to acidosis. As shown in Supplementary Figure 4b, the ATP level was steeply downregulated under acidosis. This decrease in ATP level was not recovered by ACSL5 overexpression. These results suggest that the ATP level could not be a critical factor for the ACSL5-mediated promotion of glioma cell survival under acidosis.

Upregulation of tumor-related factors by ACSL5 under extracellular acidosis conditions

To clarify the molecular mechanisms of ACSL5-dependent survival, we undertook Affymetrix GeneChip (Human Genome U133 plus 2) analysis and characterized the global program of transcription that reflects the cellular response to extracellular acidosis and the effect of ACSL5 overexpression on it. We hypothesized that extracellular acidosis could either induce a set of cell

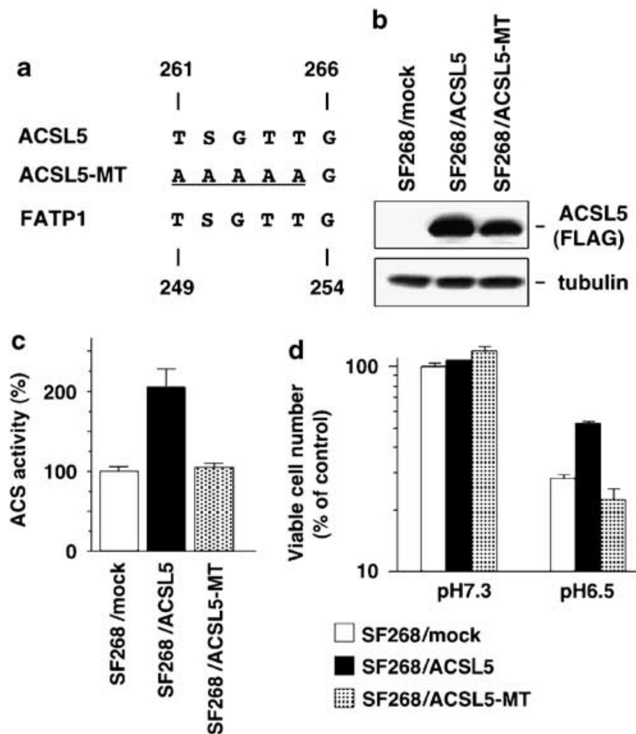

Figure 3 Acyl-CoA synthetase 5 (ACSL5) catalytic activity-dependent cell survival under extracellular acidosis conditions. (**a**) The amino-acid sequences of the putative active site in ACSL5 and FATP1. The amino-acid sequence, TSGTT (261–265), in wild-type ACSL5 was converted to AAAAA in ACSL5-MT. (**b**) The expression of FLAG epitope-tagged ACSL5 or ACSL5-MT in transduced SF268 cells as revealed by western blot with monoclonal anti-FLAG antibody. The expressions of α-tubulin were measured as loading controls. (**c**) ACS activities in ACSL5- or ACSL5-MT-transduced SF268 cells. The ACS assay was performed as described in Materials and methods. (**d**) Cells were seeded as in Figure 1c (day 0) and maintained under normal (pH 7.3) or acidic (pH 6.5) conditions. Cell numbers were counted on day 5. Data are mean values of three independent experiments, and error bars show standard deviations.

death-inducing and growth inhibitory factors or attenuate a set of genes that are required for cell survival. ACSL5 could prevent such genetic alterations. To test these hypotheses, we identified genes that are significantly induced or decreased after low pH treatment of SF268 cells. First, we extracted 229 genes in which the expression levels were altered by more than threefold during the 6-day exposure to extracellular acidosis. Second, we compared the expressions of these genes in SF268/ACSL5 cells with those in SF268/mock cells. Overall, the induction or reduction patterns were similar between the two cell lines (Supplementary Figure 5), suggesting that ACSL5 does not attenuate general stress responses to low pH but rather that some specific signals activated by ACSL5 could be involved in selective survival under low pH conditions. Therefore, we focused on genes in which the expressions were specifically regulated by ACSL5. Because ACSL5 promoted survival under acidosis conditions through its ACS catalytic activity, we tried to identify genes in which induction or decrease by ACSL5 depended on ACS catalytic activity. To determine this, we extracted genes that were up- or downregulated exclusively in SF268/ACSL5 (more than twofold) but not in SF268/ACSL5-MT cells (less than 1.3-fold over control SF268/mock cells) under extracellular acidosis conditions. As shown in Table 1, the expressions of 18 genes were significantly changed by ACSL5 overexpression. Importantly, the genes overexpressed by ACSL5 included two tumor-related genes, MDK and the melanoma cell adhesion molecule (MCAM). MDK is a growth factor frequently overexpressed in malignant tumors, and it promotes cancer cell survival (Kadomatsu and Muramatsu, 2004). MCAM is a cell surface adhesion molecule that is strongly expressed in metastatic melanoma and involved in tumorigenicity and metastasis (Xie *et al.*, 1997). Our additional GeneChip analysis further revealed that these two genes were included in a set of genes in which the expressions were significantly reduced in SNB78 cells when treated with ACSL5 siRNAs (data not shown). Meanwhile, there have been no reports that describe tumor-related function of other ACSL5-regulated genes listed here.

ACSL5-dependent expression of MDK supports glioma cell survival under extracellular acidosis conditions

We focused on the MDK and MCAM genes, because our GeneChip analysis showed that their expressions were closely linked with ACSL5, and they have been reported to be associated with the malignant phenotype of cancer. These two genes were clearly induced by ACSL5 under low pH conditions in an ACS catalytic activity-dependent manner (Figure 4a, experiment 1). Time course analysis revealed that MDK was induced by extracellular acidosis, and the expression was strongly enhanced in SF268/ACSL5 cells. On the other hand, MCAM expression was decreased under low pH, and the decrease was prevented by ACSL5 overexpression (Figure 4a, experiment 2). To confirm their expression patterns, we performed reverse transcription–PCR analysis. As shown in Figure 4b, both MDK and MCAM mRNAs were clearly induced by ACSL5 overexpression under acidic conditions. Correspondingly, when endogenous ACSL5 was decreased by specific siRNAs, the expressions of MDK and MCAM were downregulated under low pH. Western blot analysis of protein expression further confirmed that ACSL5 enhances MDK expression, especially under acidic conditions, through its catalytic activity (Figure 4c).

To determine the function of these factors in glioma cell survival under acidosis, we examined the effect of siRNA-mediated knockdown on SF268/ACSL5 cell survival under low pH conditions. As shown in Figures 5a and b, when MDK expression in SF268/ACSL5 cells was attenuated by specific siRNAs, the decrease of MDK protein was also observed. The inhibition of MDK expression markedly reduced cell viability under acidic conditions (pH 6.5) (Figure 5c), whereas it did not influence cell survival under normal conditions (pH 7.3)

Table 1 ACSL5-regulated genes in glioma SF268 cells

Probe set ID	*Gene title*	*Gene symbol*	*Experiment 1 (fold change)*[a]			*Experiment 2 (fold change)*[b]						
			pH 6.5 (day 6)			*Mock (pH 6.5)*			*ACSL5*			
			Mock	*ACSL5*	*ACSL5-MT*	*day 0*	*day 3*	*day 6*	*day 0*	*day 3*	*day 6*	
237411_at	ADAM metallopeptidase with thrombospondin type 1 motif, 6	ADAMTS6	1.00	3.67	1.08	0.66	1.14	1.00	0.93	1.77	2.24	Increased by ACSL5
209087_x_at	Melanoma cell adhesion molecule	MCAM	1.00	2.94	1.24	2.84	1.66	1.00	2.65	2.31	2.10	
209035_at	Midkine (neurite growth-promoting factor 2)	MDK	1.00	2.35	1.02	0.71	0.77	1.00	1.61	1.59	2.60	
205206_at	Kallmann syndrome 1 sequence	KAL1	1.00	2.24	1.30	1.98	1.29	1.00	4.02	3.18	3.08	
219118_at	FK506-binding protein 11, 19 kDa	FKBP11	1.00	2.14	0.95	0.57	0.64	1.00	1.75	2.42	4.23	
205100_at	Glutamine-fructose-6-phosphate transaminase 2	GFPT2	1.00	2.13	0.95	0.40	0.31	1.00	0.65	0.65	2.00	
205304_s_at	Potassium inwardly rectifying channel, subfamily J, member 8	KCNJ8	1.00	2.09	1.27	0.66	1.02	1.00	1.32	2.34	1.98	
220673_s_at	KIAA1622	KIAA1622	1.00	2.09	1.20	1.31	1.36	1.00	2.06	2.28	3.13	
209803_s_at	Pleckstrin homology-like domain, family A, member 2	PHLDA2	1.00	2.05	0.95	0.69	1.12	1.00	1.61	2.26	2.29	
234472_at	GalNAc-T13	GALNT13	1.00	0.48	1.13	1.38	1.03	1.00	0.30	0.34	0.33	Decreased by ACSL5
1555912_at	ST7 overlapping transcript 1 (antisense non-coding RNA)	ST7OT1	1.00	0.48	1.12	0.81	0.66	1.00	0.50	0.42	0.55	
219503_s_at	Transmembrane protein 40	TMEM40	1.00	0.43	1.06	0.73	1.01	1.00	0.14	0.28	0.41	
222892_s_at	Microtubule-associated protein 2	MAP2	1.00	0.42	1.01	0.79	1.06	1.00	0.21	0.29	0.40	
203108_at	G-protein-coupled receptor, family C, group 5, member A	GPRC5A	1.00	0.41	1.06	1.38	1.04	1.00	0.65	0.68	0.63	
212444_at	CDNA clone IMAGE:6025865	—	1.00	0.38	0.76	0.59	0.70	1.00	0.28	0.37	0.50	
214156_at	Myosin VIIA and Rab interacting protein	MYRIP	1.00	0.36	0.84	1.18	1.50	1.00	0.46	0.53	0.49	
235301_at	KIAA1324-like	KIAA1324L	1.00	0.27	1.03	0.86	0.86	1.00	0.38	0.42	0.54	
212094_at	Paternally expressed 10	PEG10	1.00	0.15	1.19	1.40	1.49	1.00	0.44	0.45	0.31	

Abbreviation: ACSL5, acyl-CoA synthetase 5.
[a]In experiment 1, SF268/mock, /ACSL5 and /ACSL5-MT cells were cultured under acidic (pH 6.5) conditions for 6 days. The values of relative expression changes were calculated over mock-transfected SF268 cells as a control.
[b]In experiment 2, SF268/mock and /ACSL5 cells were cultured under acidic (pH 6.5) conditions for 0, 3 and 6 days. The values of relative expression changes were calculated over SF268/mock cells at pH 6.5 at day 6 as a control.

(Supplementary Figure 6a) or under low serum conditions (Supplementary Figure 6b). By contrast, the knockdown of MCAM did not influence cell viability under either normal or acidic pH (data not shown).

Collectively, these results indicate that ACSL5 is functionally involved in glioma cell survival under acidic tumor microenvironment. Our data further revealed that ACSL5-dependent expression of MDK is a critical factor for survival.

Discussion

Extracellular acidosis is an important factor in the malignant progression of tumors (Rofstad *et al.*, 2006), and tumor cells must develop resistance to this stress-induced cytotoxicity. Under tumor microenvironmental stresses, the defect in the p53 tumor suppressor protein is a critical factor for apoptosis resistance and cancer cell survival (Soengas *et al.*, 1999). However, low pH stress inhibits cell growth in a p53-independent manner, suggesting the involvement of other mechanisms (Reichert *et al.*, 2002). Our results suggest that enhanced cell survival by ACSL5 under low pH conditions could have a function in the progression of cancer.

Predominant function for ACSL5 in glioma cell survival
Elevated levels of fatty acid metabolism have a critical function in the malignant growth of tumors (Menendez and Lupu, 2007). Among fatty acid metabolic enzymes, ACS members catalyse an essential step in both the catabolic pathway for fatty acid degradation through the β-oxidation system and the anabolic pathway for cellular lipid synthesis (Coleman *et al.*, 2002). In this study, we showed that ACSL5 was involved in the promotion of glioma cell survival under extracellular acidosis conditions. In human glioma, aberrations are frequently observed on chromosome 10q25.1–q25.2, on which the ACSL5 gene is located and, in fact, the ACSL5 overexpression is highly correlated with malignancy of the tumors (Yamashita *et al.*, 2000). We further sequenced the ACSL5 gene in human glioma cell lines that overexpress ACSL5. We found that wild-type ACSL5 is overexpressed in A1207 and A172 cell lines (unpublished data). In the ACSL5 gene extracted from SNB78 cells, we found one amino-acid difference (M182V) when it was compared with the reported wild-type human ACSL5 gene (data not shown). However, this sequence is not conserved among species, indicating that this amino-acid sequence is not essential for functional ACS activity. These data indicate that

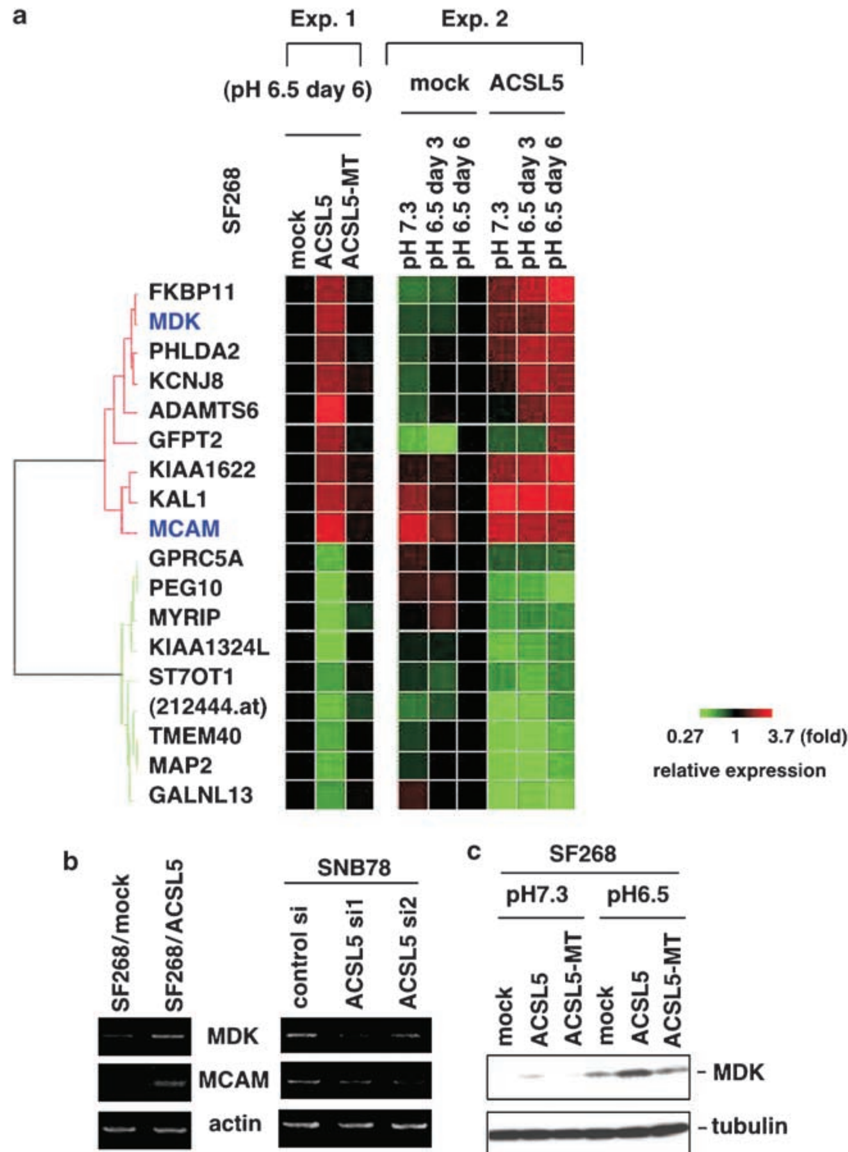

Figure 4 Identification of acyl-CoA synthetase 5 (ACSL5)-regulated gene expression signature by cDNA microarray analysis. (**a**) Hierarchical clustering using log-transformed relative expression changes over control for genes up- or downregulated exclusively in SF268/ACSL5 cells but not in SF268/ACSL5-MT cells. We applied the arbitrary cutoffs of >2-fold up- or downregulation. Each row and column represents genes and treatment conditions of cells. The values of relative expression changes were calculated over SF268/mock (pH 6.5, day 6) as a baseline. The data in the three left columns and in the six right columns are derived from independent experiments. In each experiment, duplicate samples were analysed. (**b**) ACSL5-dependent regulation of midkine (MDK) and melanoma cell adhesion molecule (MCAM) mRNA expressions. SF268/mock and SF268/ACSL5 cells were cultured for 6 days under acidic conditions (pH 6.5). SNB78 cells were treated with ACSL5 siRNAs or control siRNA and cultured for 48 h under acidic conditions (pH 6.5). Total RNAs were then prepared, and the expressions of MDK and MCAM were analysed by reverse transcription (RT)–PCR. (**c**) ACSL5-dependent regulation of MDK protein expression. SF268/mock, SF268/ACSL5 and SF268/ACSL5-MT cells were cultured for 6 days under normal (pH 7.3) or acidic (pH 6.5) conditions. Cell lysates were prepared, and the expressions of MDK were detected by an anti-MDK antibody. The expressions of α-tubulin were measured as loading controls.

functional ACSL5 is overexpressed in glioma and could have an essential function in glioma cell survival. We have shown earlier that inhibiting multiple ACS activities strongly induces apoptosis, whereas this cell death is almost completely suppressed by a single gene transfer of ACSL5 (Mashima *et al*., 2005). In addition, among mammalian ACS, only ACSL5 restores the growth of an *Escherichia coli* strain that lacks FadD, the only known ACS enzyme in the *E. coli* (Caviglia *et al*., 2004). These observations suggest that among ACS members, ACSL5 could have a predominant function in cell survival.

As we have shown, ACSL5 confers selective survival advantage under acidosis conditions but not under other tumor microenvironment stresses. Although we showed that *in vivo* treatment with ACSL5 siRNA significantly suppressed the growth of A1207 tumor (Supplementary Figure 3d), it is still not clear whether

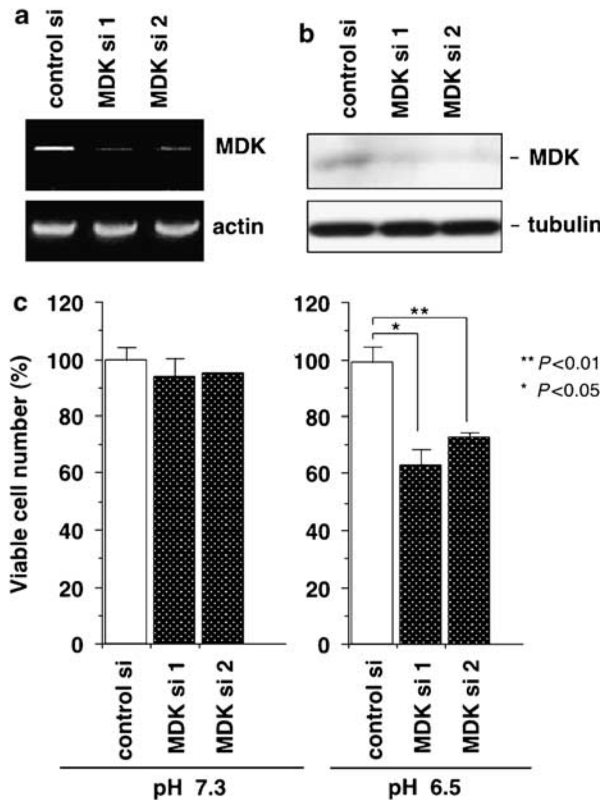

Figure 5 Involvement of midkine (MDK) in acyl-CoA synthetase 5 (ACSL5)-mediated glioma cell survival under extracellular acidosis conditions. (**a**) mRNA expression of MDK in SF268/ACSL5 cells treated with siRNAs. Cells were treated with stealth siRNAs that targeted MDK, or with control siRNA, as described in Materials and methods. At 48 h after siRNA treatment, total RNAs were prepared and the expressions of MDK were analysed by reverse transcription (RT)–PCR. The expressions of β-actin were also analysed as loading controls. (**b**) Protein expression of MDK in SF268/ACSL5 cells treated with siRNAs. At 48 h after siRNA treatment, cell lysates were prepared and the expressions of MDK were detected by an anti-MDK antibody. The expressions of α-tubulin were also measured as loading controls. (**c**) Viability of SF268/ACSL5 cells after MDK knockdown under normal and acidic conditions. At 24 h after siRNA treatment, SF268/ACSL5 cells were cultured under normal (pH 7.3) or low pH (pH 6.5) conditions for 4 days, and viable cell numbers were counted. Data are mean values of three independent experiments, and error bars show standard deviations. *P*-values (two-sided) were calculated using the Student's *t*-test. *P*-values of <0.05 were considered statistically significant. $^{**}P<0.01$; $^{*}P<0.05$.

the expression of ACSL5 alone could be enough to promote tumor growth or survival *in vivo*. To address these questions, we established a tumorigenic U87MG glioma cell line that stably overexpressed ACSL5 and implanted U87MG/mock and U87MG/ACSL5 cells subcutaneously in nude mice. As a result, we did not observe significant advantage of tumor growth in ACSL5-overexpressed U87MG tumors (data not shown). These data suggest that cooperation of ACSL5 with other survival factors could further be required for promotion of glioma growth *in vivo* where several types of stress would coexist.

Selective induction of MDK gene by ACSL5 under low pH conditions

Our study showed that ACSL5 is responsible for the expression of some tumor-related factors. Among them, the ACSL5-dependent expression of MDK was critical for survival under acidic conditions. Importantly, the ACSL5-dependent expression of MDK was strongly augmented by low pH stress (Figures 4a and c). This could explain the selective involvement of ACSL5-mediated MDK induction in glioma cell survival under low pH conditions. ACSL5 affects intracellular fatty acid levels through its catalytic activity. These changes may trigger signaling pathways that lead to MDK induction, as fatty acids act as specific ligands for some nuclear receptors, such as peroxisome proliferator-activated receptor (PPAR) (Schoonjans *et al*., 1996). Although the promoter region of the MDK gene does not possess any direct responsive element for PPAR, it does contain specific elements, including the steroid/thyroid hormone receptor-binding site (TRE) (Uehara *et al*., 1992). Because PPAR can form a heterodimer with a thyroid hormone receptor (Bogazzi *et al*., 1994), the element might have a function in the ACSL5-dependent induction of the MDK gene. Our GeneChip microarray analysis revealed that the expression of ACSL5 was not significantly induced under acidic culture conditions (data not shown). These data suggest that although ACSL5 induces the expression of MDK, the acidosis-dependent induction of MDK would be caused by another mechanism.

Cancer cell survival and growth arrest by MDK

Several reports have indicated that MDK has a crucial function in the survival and malignant phenotype of cancer (Kadomatsu *et al*., 1997; Takei *et al*., 2001; Kadomatsu and Muramatsu, 2004). MDK also confers chemotherapy resistance to cancer cells (Mirkin *et al*., 2005). Considering the multiple functions of this growth factor, ACSL5-dependent expression of MDK may have a function not only in cell survival under acidosis but also in other malignant phenotypes of cancer cells. Our data indicated that ACSL5 induces MDK expression and concomitantly promotes cell cycle arrest at the G1 phase, especially under extracellular acidosis (Supplementary Figure 2c). It was recently reported that MDK overexpression also promotes cell cycle arrest at the G1 phase (Mirkin *et al*., 2005). These observations suggest that cell cycle arrest caused by the ACSL5-induced MDK could be important for survival under stress conditions. In fact, G1 arrest is known to be antagonistic to stress-induced cytotoxicity (Knudsen *et al*., 2000).

Other factors affected by ACSL5

We identified MCAM as another factor regulated by ACSL5. Although our data did not show its function in glioma cell survival under low pH, MCAM could have a function in other malignant phenotypes such as tumor metastasis (Xie *et al*., 1997). Our GeneChip analysis also identified G-protein-coupled receptor C2A (GPRC5A) as a gene selectively downregulated by ACSL5 (Table 1).

GPRC5A was recently reported as a lung tumor suppressor (Tao *et al*., 2007). In the present analysis, we did not focus on this gene, as its expression was not clearly upregulated in SNB78 cells that were treated with ACSL5 siRNAs (data not shown). Recently, it was shown that ACSL5 partitions exogenously derived fatty acids toward triacylglycerol synthesis and storage (Mashek *et al*., 2006). The function of this pathway in ACSL5-mediated glioma cell survival should be examined in future studies.

Global view of the low pH-induced gene expression signature

We showed that the reduced glioma cell viability under low pH conditions was not derived from caspase-dependent, typical apoptosis (Kitanaka and Kuchino, 1999). Although the mechanisms of the reduced cell viability are still unknown, our analysis identified a set of genes that is highly induced or decreased by low pH stress. These genes included cell death regulators, metastasis suppressors and stress-responsive genes (data not shown). The function of these genes in stress-induced toxicity is still to be clarified.

Conclusions: ACS as a molecular target for cancer therapy

Emerging evidence has identified fatty acid metabolisms as promising molecular targets for cancer therapeutics. Among them, ACS members are candidate molecules to induce cancer-selective cell death (Cao *et al*., 2000; Mashima *et al*., 2005). Our present data indicate the critical function of ACSL5 in glioma cell survival and suggest that this enzyme could be a rational therapeutic target. On the other hand, our analysis revealed that glioma cells also express other ACS isozymes, including ACSL1, 3 and 4 (data not shown), the functions of which in tumor survival are still unknown. Further analysis including the effect of simultaneous inhibition of multiple ACS isozymes on the survival of cancer could open the door for novel ACS-targeted cancer therapy.

Materials and methods

Cell lines, cell culture and measurement of growth inhibition

Human glioma SF268 and SNB78 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum. Human glioma A1207 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Mishima *et al*., 2001). To examine the effect of extracellular acidosis, the culture medium was acidified by supplementing the regular medium with 25 mM HEPES and adjusting the acidity to a final pH of 6.5 with 0.5 N HCl, as described earlier (Ohtsubo *et al*., 1997). We measured pH of the medium before and after treatment. Changes in pH were not observed after cells were treated. To estimate the effect of changes in ionic balance and osmolality after the addition of HCl, we added the same concentration ($\sim$20 mM) of NaCl to the medium as a control. We found no significant effect of the NaCl addition on glioma cell growth. Hypoxic conditions were achieved using an anaerobic chamber and BBL GasPac Plus (Becton Dickinson, Cockeysville, MD, USA), which catalytically reduces oxygen levels to less than 10 p.p.m. within 90 min (Seimiya *et al*., 1999). To achieve low serum conditions, we cultured cells in the medium containing 0.1% fetal bovine serum. Cell viability under low pH, hypoxia and low serum or after treatment with siRNA was evaluated by counting viable cells using a hemocytometer. The cell viability was determined by Trypan blue exclusion. Statistical evaluations were performed using Student's *t*-test. *P*-values of <0.05 were considered statistically significant.

Vector construction and retrovirus-mediated gene transfer

For the expression of human ACSL5, pHa-ACSL5-FLAG-IRES-DHFR was constructed as described earlier (Mashima *et al*., 2005). To construct an inactive mutant of ACSL5 (ACSL5-MT), we referred to the construction of inactive fatty acid transport protein (FATP1), a very long chain ACSL (Coe *et al*., 1999). In the case of FATP1, a six-amino-acid substitution into the putative active site (amino acid 249–254: TSGTTG) was enough to inactivate its acyl-CoA synthetase. As ACSL5 also possesses a putative active site with the same sequence (amino acid 261–266: TSGTTG), we converted the amino-acid TSGTT (261–265) to AAAAA to generate pHa-ACSL5-MT-FLAG-IRES-DHFR using a Quik-Change XL site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Retrovirus-mediated gene transfer of pHa-IRES-DHFR (mock), pHa-ACSL5-FLAG-IRES-DHFR or pHa-ACSL5-MT-FLAG-IRES-DHFR constructs was performed as described earlier (Mashima *et al*., 2005).

siRNA treatment

siRNA oligonucleotides to ACSL5 were synthesized by Dharmacon Research Inc. (Lafayette, CO, USA). The two siRNAs tested were targeted to the 5′-GCACCAGAGAAGA UAGAAA-3′ (siRNA 1) and 5′-GUGCACUGCUUGUGAG AAA-3′ (siRNA 2) sequences of the human ACSL5 mRNA. As a control, we purchased a nonspecific control duplex (5′-ACUCUAUCUGCACGCUGACUU-3′) from Dharmacon Research Inc. The stealth siRNA oligonucleotides to MDK were synthesized by Invitrogen (Carlsbad, CA, USA). The two siRNAs tested for MDK were 5′-UGAGCAUUGUAGCGC GCCUUCUUCA-3′ (siRNA 1) and 5′-AUUGAUUAAAG CUAACGAGCAGACA-3′ (siRNA 2). A negative universal control siRNA (medium no. 2) was purchased from Invitrogen. siRNAs were transiently introduced into the cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Western blot analysis

Western blot analysis was performed as described earlier (Mashima *et al*., 2005) with the following primary antibodies: mouse anti-FLAG (M2; Sigma), mouse anti-α-tubulin (Sigma), mouse anti-ACSL5 (Abnova, Taipei, Taiwan) or rabbit anti-MDK (Abcam, Cambridge, UK).

Measurement of ACS activity

Total cell lysates were prepared and ACS activity was measured as described earlier (Mashima *et al*., 2005).

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