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Functions of the breast cancer resistance protein (BCRP/ABCG2) in chemotherapy☆

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ABSTRACT

The breast cancer resistance protein, BCRP/ABCG2, is a half-molecule ATP-binding cassette transporter that facilitates the efflux of various anticancer agents from the cell, including 7-ethyl-10-hydroxycamptothecin, topotecan and mitoxantrone. The expression of BCRP can thus confer a multidrug resistance phenotype in cancer cells, and its transporter activity is involved in the *in vivo* efficacy of chemotherapeutic agents. Thus, the elucidation of the substrate preferences and structural relationships of BCRP is essential to understanding its *in vivo* functions during chemotherapeutic treatments. Single nucleotide polymorphisms (SNPs) have also been found to be key factors in determining the efficacy of chemotherapeutics, and those therapeutics that inhibit BCRP activity, such as the SNP that results in a C421A mutant, may result in unexpected side effects of the BCRP- anticancer drugs interaction even at normal dosages. In order to modulate the BCRP activity during chemotherapy, various compounds have been tested as inhibitors of this protein. Estrogenic compounds including estrone, several tamoxifen derivatives in addition to phytoestrogens and flavonoids have been shown to reverse BCRP-mediated drug resistance. Intriguingly, recently developed molecular targeted cancer drugs, such as the tyrosine kinase inhibitors imatinib mesylate, gefitinib and others, can also interact with BCRP. Since both functional SNPs and inhibitory agents of BCRP modulate the *in vivo* pharmacokinetics and pharmacodynamics of its substrate drugs, BCRP activity is an important consideration in the development of molecular targeted chemotherapeutics.

Contents

1. BCRP and Cancer

Cancer drug resistance is a major problem in clinical chemotherapy such that overcoming multidrug resistance to functionally and structurally unrelated anti-cancer agents is of critical importance for future treatments using these molecules and their derivatives. Various

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mechanisms, such as reduced drug uptake, the efflux of intracellular drugs, the activation of DNA repair pathways, and the induction of anti-apoptotic machineries can confer multidrug resistance in cancer cells [1]. Among these mechanisms, the ABC transporter proteins, particularly P-glycoprotein (ABCB1), have been extensively examined as key components in pathways that result in a multidrug resistant phenotype [2–4]. A second ABC transporter protein, the breast cancer resistance protein (BCRP/ABCG2), is a 655 amino acid protein that contains an ATP-binding domain and six transmembrane domains, and it is a half transporter member of the ABCG subfamily [5]. BCRP was originally identified in anticancer drug-resistant human cancer cell lines obtained by *in vitro* selection [6–8].

In a similar fashion to the well-studied multidrug resistant protein P-glycoprotein, which is known to play important roles in the multidrug resistant phenotype of clinical cancer cells [9,10], the overexpression of BCRP renders cancer cells resistant to various chemotherapeutic drugs such as the topoisomerase I inhibitor topotecan and the antifolate agent methotrexate [8,11]. In a previous study from our laboratory, we observed that BCRP-transduced human myelogenous leukemia K562 cells (K562/BCRP) show a 24-fold higher resistance to SN-38, a 10-fold higher resistance to mitoxantrone, and a 10-fold higher resistance to topotecan [12]. However, the P-glycoprotein substrates vinblastine, paclitaxel, and verapamil are not eliminated by BCRP. As a half transporter, BCRP functions as a homodimeric/oligomeric efflux pump [13,14], and in a manner that is similar to other ABC transporters, BCRP eliminates a variety of compounds such as sulfated hormone metabolites, the chlorophyll metabolite pheophorbide A, fluorescent dyes such as Hoechst 33342 and BODIPY-prazosin, cimetidine, various flavonoids, some antibiotics, and various cytotoxic agents [15,16]. Typical BCRP substrates such as irinotecan and SN-38 are detoxified by glucuronidation via the activity of UDP-glucuronyltransferase, although BCRP can eliminate SN-38-glucuronide even though this substrate has a much lower affinity for BCRP than SN38 itself [17]. Interestingly, BCRP can also transport another of the glucuronideconjugates, 17-β-estradiol 17-(β-D-glucuronide) in addition to the sulfated-conjugates estrone-3 sulfate and dehydroepiandrosterone [18,19]. Our previously reported results of a membrane vesicle assay using ^{3}H-labeled compounds indicated that the ^{3}H-labeled estrone sulfate, but not the ^{3}H-labeled estrone or estradiol, was imported by membrane vesicles prepared from K562/BCRP cells in an ATP-dependent manner [20]. Hence, BCRP appears to transport xenobiotics and endogenous metabolites of steroids that are either sulfated or glucuronidated.

It has been suggested that the expression of BCRP is associated with a poor response to cancer chemotherapy and may be responsible for clinical drug resistance [21–23]. Moreover, BCRP is widely expressed in normal cells and tissues including the capillary endothelial cells, the hematopoietic stem cells [24,25], the maternal–fetal barrier of the placenta and the blood-brain barrier [26]. BCRP also appears to play a protective role against xenobiotics and their metabolites [15,19]. The apical localization of BCRP in the intestinal epithelium and in the bile canalicular membrane also suggests the intestinal absorption and hepatobiliary excretion of BCRP substrates [26–29]. Thus, BCRP may restrict the bioavailability of orally administered anticancer agents that are BCRP-substrates such as topotecan (and its metabolite SN-38), irinotecan, camptotecin derivatives, methotrexate, and flavopiridol, in addition to other compounds [16]. In this regard, the dual inhibitor of BCRP and P-glycoprotein, GF120918, has been reported to increase the oral bioavailability of topotecan through the inhibition of BCRP function [30]. Since undesired toxic effects of chemotherapeutic drugs on the digestive organs are a significant problem during clinical cancer chemotherapy, the functional activity of BCRP is an important consideration for BCRP-transportable drug pharmacokinetics in patients.

It has been suggested that BCRP underlies the drug resistance observed in clinical samples of different cancers such as acute myelogenous leukemia (AML) and acute lymphocytic leukemia (ALL) [21–23,31–34]. In addition, BCRP expression in solid tumors has been examined [35]. However, there is some controversy surrounding the precise roles of BCRP as functional correlations between BCRP expression and clinical drug resistance have not yet been definitively demonstrated. Since P-glycoprotein expression is also an important factor in the drug resistance levels of clinical leukemia, a larger scale analysis that assesses both BCRP and P-glycoprotein will be required to properly delineate the involvement of BCRP expression in the drug resistance of cancer patients. Furthermore, in addition to expression profiling, recent advances in ABC transporter research have highlighted that the activity of these factors is affected by both posttranslational regulation [36,37] and genetic polymorphisms [38–40]. Thus, in order to elucidate the relationship between the functions of BCRP and the clinical outcomes following chemotherapy, new and more sensitive methods for testing specific pharmacological inhibitors, and also immunological and genetic probes, will be required.

2. BCRP structure and anti-cancer drugs

Structural and functional studies of BCRP and its substrates have provided valuable insights into the molecular mechanisms underlying BCRP-mediated multidrug resistance. Intriguingly, the cloning of BCRP cDNAs from drug-selected cells and normal tissues have also uncovered functional variations associated with amino acid substitutions in the BCRP protein resulting in an alteration in substrate preferences. BCRP proteins expressed in drug-selected cells such as those of the S1-M1-80 and MCF7/AdVp3000 cell lines were unexpectedly found to be mutant forms, and several unique mutations at amino acid position 482 in BCRP have been identified [41]. MCF7/AdVp3000 and S1-M1-80 cells expressing R482T and R482G variants of BCRP, respectively, are highly resistant to both mitoxantrone and doxorubicin. Anthracyline resistance and a rhodamine efflux ability are also unique phenotypes in these two cell lines when they are overexpressing BCRP [41,42]. Subsequently, we have learned that substitutions of Arg with either Gly or Thr at position 482 in BCRP confer an additional efflux activity against rhodamine 123, doxorubicin, and other anthracyclins, which are not substrates for wild-type BCRP [41,43]. Moreover, the BCRP variants R482G and R482T lose their methotrexate-transporting activity but at the same time confer increased mitoxantrone resistance [18,42,44,45]. These findings suggest that structural variations of BCRP can strongly influence its drug efflux functions and substrate preference. A positively charged Arg at position 482 affects the interaction between BCRP and the drug, and therefore the COOH-terminus of the transmembrane (TM) 3 region that is in close proximity to position of amino acid 482 appears to be involved in the substrate-binding pocket interface of BCRP [45–47].

In the case of P-glycoprotein, extensive mutagenesis studies and recent three-dimensional structural analyses have suggested that its transmembrane domains are involved in influencing its substrate-specificity [9,48,49]. Hence, in order to further elucidate the structural features of BCRP and how these features relate to substrate recognition, systematic mutational analysis of its TM regions would likely be of great benefit and provide valuable information regarding the molecular mechanisms underlying multidrug interactions. In an analogous manner to studies of P-glycoprotein, we performed such mutational analysis of BCRP using 32 mutants of this protein and found that Glu 446 in TM2, Arg 482 in TM3, Asn 557 in TM5, and His 630 in TM6 alter its drug resistant phenotype [50]. These findings confirmed that the transmembrane region of BCRP plays important roles in its activity. Moreover, murine fibroblast PA317 cells expressing E446 mutant BCRPs did not show drug resistance to either mitoxantrone or SN-38.

Furthermore, in a manner similar to that observed in S1-M1-80 and MCF7/AdVp3000 cells, 13 variant BCRPs harboring an amino acid substitution at R482 (R482N, C, M, S, T, V, A, G, E, W, D, Q and H, but not Y or K) conferred strong resistance to doxorubicin and mitoxantrone in

PA317 cells. Mutations in BCRP at positions N557 and H630 however severely affected this resistant phenotype. Cells expressing either the N557D or the H630E BCRP mutant displayed a lower resistance to SN-38, although the mitoxantrone-resistance of these cells was comparable to that observed for the wild-type BCRP-expressing cells. Consistently, recent structural studies using three-dimensional homology modeling of BCRP have suggested that the transmembrane domains of BCRP function as a drug-recognition interface [46,47]. These data, coupled with other numerous studies regarding BCRP functions, indicate that the drug efflux activity of this protein appears to be influenced by various mutations that will necessarily affect the clinical efficacy of BCRP-transportable anticancer drugs.

3. Effects of BCRP SNPs upon drug resistance

As mentioned in the previous section, amino acid variations in BCRP may be associated with its drug-transporter function. In addition, a variety of germ-line mutations in the *BCRP* gene have been found in ethnically diverse populations [39,51–53]. Such variations, particularly of the single nucleotide polymorphisms (SNPs) in the *BCRP* genomic region should be evaluated to estimate the possible effects of BCRP among different patients.

3.1. C421A (Q141K) BCRP SNP

In a previous study, we screened for BCRP SNPs among a population of Japanese individuals and in human cancer cell lines where we identified three variant *BCRP* cDNAs harboring the following substitutions: G34A (V12M), C421A (Q141K) and an amino acid deletion of residues 944-949 that lacks Ala-315 and Thr-316 (Δ315-6) [54]. The G34A and C421A variations were determined to be SNPs, and we have subsequently determined that the C421A *BCRP*-transfected murine fibroblast PA317 (PA/Q141K) cells show lower exogenous BCRP protein levels than the wild-type *BCRP*-transfected cells [54]. The intracellular topotecan accumulation in PA/Q141K cells was also found to be higher compared with other *BCRP* transfectants, indicating that the C421A (Q141K) SNP influences BCRP function. This polymorphism is located within the functionally important ATP-binding region between the Walker A and B motifs of BCRP and likely affects its ATPase activity levels, since the ATPase activity of the membrane of C421A *BCRP*-transduced insect Sf9 cells were 1.3-fold lower than that of the wild-type BCRP transduced cells [38,55]. Regarding the Q141K BCRP SNP, there are conflicting reports on its effects upon expression levels, localization, and functionality [38,54,56–58]. Additional studies will be required to clarify the mechanism by which the Q141K mutation reduces the protein expression levels of BCRP. In contrast to the above results, the G34A *BCRP*-transfected PA317 (PA/V12M) cells showed comparable protein expression levels and drug resistance levels to the wild-type *BCRP*-transfected cells.

Our earlier studies on the frequency of the C421A SNP in a normal Japanese population showed that 57 of 124 samples possessed the A421 allele and that 9 of these were homozygous for this polymorphism [39,59]. These data indicate that some Japanese individuals likely express low amounts of BCRP. Furthermore, the C421A SNP is of some importance as the allelic frequency of this variant differs greatly between diverse populations. This SNP appears to be very common in Asian populations, with reported allelic frequencies between 27% and 34% [38,54,57]. In contrast, the C421A SNP is rare in sub-Sahara African and in African American populations, with a frequency of <5% [60]. Its frequency in Caucasian populations is approximately 10% [61]. The physiological significance of the C421A-*BCRP* SNP has also now been evaluated in relation to the pharmacokinetics of diflomotecan, a new anticancer agent that is a derivative of camptothecin, during a phase I study [62]. In this analysis, 5 patients who were heterozygous for the A421 allele showed much higher plasma levels of diflomotecan after intravenous administration compared with 15 wild type individuals who were homozygous for the allele (mean values of 138 ngxh/mLxmg^{-1} versus 46.1 ngxh/mLxmg^{-1}, respectively). The findings from this clinical study indicate that the expression levels and functions of the BCRP derived from the C421A-*BCRP* allele are adversely perturbed in comparison to the wild-type allele. Hence, the C421A SNP is considered to be one of the most important BCRP variations in terms of cancer chemotherapy and drug resistance.

3.2. C376T (Q126stop)-BCRP SNP

We have identified another SNP within the *BCRP* gene, C376T, which substitutes a stop codon for Gln-126 (Q126stop) and is present at a low frequency in samples from healthy Japanese individuals as a heterozygote (reported frequencies of 3/124 and 2/120 in two studies, respectively) [54,60]. Similar stop codon SNPs have been reported in the *MRP2* gene and are linked to the rare hyperbilirubinemia associated with Dubin-Johnson syndrome [63]. Although the frequency of the T376 allele of *BCRP* is low and has not been observed in Caucasian or African American groups, a combination of the C376T and C421A SNPs would be expected to occur at a significant rate in the Japanese population. Since these SNPs are each anticipated to have negative effects on BCRP activity, the combined C376T/C421A variants would be expected to show severely reduced BCRP activity. Such individuals may thus be hypersensitive to anticancer agents.

3.3. Additional BCRP SNPs

The *BCRP* SNPs identified to date are summarized in Table 1. These polymorphisms include G34A, G151T, C376T, C421A, C458T, C496G, A616C, T623C, T742C, G1000T, T1291C, T1465C, A1768T and G1858A, all of which generate amino acid substitutions. However, additional mutations such as T114C, C369T, C474T, A564G, G1098A, and A1425G have been identified in the coding region of *BCRP*. Among these SNPs, with the exception of C376T and C421A, only a few have been studied

Table 1
Identified SNPs within the BCRP gene

Variation	Effect	Domain
A-1379G	-	-
Δ-654/-651	-	-
G-286C	-	-
T-476C	-	-
Δ-235A	-	-
A-113G	-	-
A-29G	-	-
G34A	V12M	N-terminal
T114C	No change	N-terminal
G151T	G51C	N-terminal
C369T	No change	NBD
C376T	Q126stop	NBD
C421A	Q141K	NBD
C458T	T153M	NBD
C474T	No change	NBD
C496G	Q166E	NBD
A564G	No change	NBD
A616C	I206L	NBD
T623C	F208S	NBD
T742C	S248P	Linker
G1000T	E334stop	Linker
G1098A	No change	Linker
T1291C	F431L	TMD
A1425G	No change	TMD
T1465C	F489L	TMD
A1768T	N590Y	TMD
G1858A	D620N	TMD
G2237T	-	-
G2393T	-	-

NBD, nucleotide-binding domain; TMD, transmembrane domain.

in association with the protein expression levels and function of BCRP. The G34A SNP generating an amino acid substitution at position 12 (V12M) has been observed in the Japanese population [54]. The highest allele frequency for this polymorphism is observed in Mexican-Indians, and there are significant differences in the frequencies of this SNP between Caucasian, Japanese and Swedish populations [56,64]. Transfection studies of the V12M BCRP have shown, however, that the expression levels and drug-resistance associated with this variant are comparable to the wild type BCRP and therefore that this SNP has no significant impact on the BCRP protein activity [54]. It is noteworthy, however, that, although the physiological and pathological significance of the G34A SNP in the *BCRP* gene is unclear, a recent report has suggested a possible association between this polymorphism and alternative splicing event of the BCRP mRNA, specifically in the splicing of the liver-specific polymorphic exon 2 of these transcripts [65]. Polymorphic and differential expression of alternatively spliced BCRP mRNA involving exon 1b is also observed in the liver [66] and appears to be associated with lower BCRP expression. Approximately 90% of the cases characterized by a G34A BCRP SNP display exon 2 skipping in the liver that may suggest that the lower expression of BCRP transcripts in this organ may be associated with this SNP in the Hispanic population [65].

4. BCRP and anticancer kinase inhibitors

Recent advances in molecular targeted therapy have resulted in the development of various anticancer drugs with unique pharmaceutical properties [1]. In particular, a growing number of small-molecule protein kinase inhibitors have been brought into clinical use and have shown great potential as anticancer drugs [67,68]. Imatinib mesylate was the first protein kinase inhibitor to be approved as an anticancer drug and targets BCR-ABL, the platelet-derived growth factor receptor (PDGFR), and stem cell factor/c-kit [69]. Imatinib is highly effective against chronic myeloid leukemia and other cancers associated with deregulation of kinase pathways. Resistance to this drug is typically conferred by mutations arising in the target kinase within the drug-kinase-interaction region [70–73]. Interestingly, another mechanism leading to imatinib resistance has been proposed and involves a correlation with P-glycoprotein expression [74,75]. As shown in Table 2, a number of recent studies have indicated a possible interaction of several kinase inhibitory drugs with ABC transporters, including P-glycoprotein and BCRP [76–81]. Among members of the ABC transporter family, BCRP seems to have a strong tendency to interact with clinically important kinase inhibitors including imatinib [78–80,82–85], nilotinib [86], gefitinib [52,87–93], canertinib [94], erlotinib [95,96], and lapatinib [97]. Imatinib and nilotinib are both inhibitors of BCR-ABL, whereas gefitinib, erlotinib, canertinib, and lapatinib target the HER family. Functional and pharmaceutical interactions between BCRP and imatinib or gefitinib have been extensively examined, and these data indicate that, although these kinase inhibitors are substrates for BCRP, they exhibit potent inhibitory activity against this ABC transporter when used at relatively high concentrations [86].

Table 2
Functional interaction between tyrosine kinase inhibitors and BCRP

	BCRP	P-glycoprotein	Ref
Imatinib	+	+	[78,79,82]
Nilotinib	+	+	[86]
Dasatinib	ND	+	[102]
INNO-406	ND	-	[101]
Gefitinib	+	+	[78,87,90,92,93]
Erlotinib	+	+	[95,96,107]
Canertinib	+	ND	[94]
Flavopiridol	+	+	[109]

ND, not determined.

4.1. BCR-ABL kinase inhibitors

Imatinib mesylate is a tyrosine kinase inhibitor of BCR-ABL, PDGFR, and c-Kit that is now a widely used anticancer drug. In terms of the functional interaction between this agent and ABC transporters, contentious observations were reported in initial studies regarding whether or not imatinib is in fact a substrate of these efflux proteins. Recent analyses by Brendel et al. [86] have further shown that BCRP-expression confers imatinib-resistance and reduces imatinib accumulation in K562 cells, effects that are abrogated by the BCRP inhibitor fumitremorgin C (FTC). However, this previous study also demonstrated that imatinib directly interacts with BCRP at the substrate-interacting region and can stimulate its ATPase activity. Intriguingly, this study suggests that imatinib-transportation by BCRP may be concentration dependent as the efflux of this drug by BCRP was facilitated when imatinib was at low concentrations (<1 μM). At relatively high concentrations (μM level), imatinib has also been observed as a potent BCRP inhibitor that reverses the BCRP-mediated drug resistance to SN-38 and topotecan [79] and increases mitoxantrone accumulation in BCRP-expressing CD34+ cells [84]. Furthermore, *in vivo* studies indicate that BCRP, together with P-glycoprotein, appears to regulate the penetration of imatinib into brain tissue. Imatinib brain penetration in *Bcrp1* knockout mice was found to increase [80], whereas the inhibition of BCRP and P-glycoprotein activities significantly improved the brain penetration of imatinib in wild-type mice [98]. These observations support the notion that imatinib can function as both a substrate for and inhibitor of BCRP.

Nilotinib is a newly developed BCR-ABL kinase inhibitor with improved selectivity and potency [99,100]. BCRP-expressing K562 cells showed nilotinib resistance over a narrow range of concentrations (10 to 25 nM) [86] such that nilotinib resistance by BCRP may not be a significant phenomenon in the clinical setting. However, in this same study, BCRP expression was found to reduce the intracellular accumulation of nilotinib, a compound that binds to BCRP and stimulates its ATPase activity [86]. In addition, nilotinib reverses the BCRP-mediated Hoechst 33342 dye exclusion and therefore appears also to be both a substrate and a potent inhibitor of BCRP. Functional interactions between P-glycoprotein and the novel BCR-ABL inhibitors dasatinib and INNO-406 have also been reported [101,102], but the effects of these agents against BCRP have not yet been examined.

4.2. EGFR/HER kinase inhibitors

Gefitinib is an orally active, selective epidermal growth factor receptor-tyrosine kinase inhibitor used in the treatment of patients with advanced non-small cell lung cancer [103,104]. Human epidermoid carcinoma A431 cells and human non-small cell lung cancer PC-9 cells, both of which are highly sensitive to gefitinib, were found to become resistant to gefitinib at nanomolar concentrations upon transduction with BCRP [52]. Consistent with this observation, Elkind et al. [90] have also reported that the expression of BCRP confers gefitinib-resistance in A431 cells. However, it should be noted from these studies that BCRP expression did not confer ectopic gefitinib-resistance to naturally occurring gefitinib-insensitive cells as the efflux function of BCRP itself seems to be suppressed by relatively high concentrations (~μM level) of this drug. In this regard, studies from our laboratory and others have demonstrated that BCRP overexpression in the less gefitinib-sensitive cell lines K562, P388, and MCF7 was not in fact a determinant of gefitinib sensitivity [88,91]. In contrast, we have shown from our analyses that gefitinib can reverse the SN-38 resistant phenotype in human leukemia K562/BCRP and mouse leukemia, P388/BCRP cells, and suppress the ATP-dependent transport of estrone sulfate in membrane vesicles prepared from K562/BCRP cells [88]. Co-treatments with gefitinib have also been shown to induce the intracellular accumulation of topotecan in K562/BCRP cells, and the combination of irinotecan with gefitinib resulted in a potent

enhancement of irinotecan cytotoxicity in multiple tumor models [87,88,91]. These results indicate that gefitinib can inhibit the transporter activity of BCRP and thus reverse the BCRP-mediated drug resistance when used at relatively high concentrations in the micromolar range.

With regard to possible interactions between BCRP and gefitinib *in vivo*, Stewart et al. [87] have demonstrated that the oral bioavailability of irinotecan is affected by the oral administration of gefitinib. BCRP has been detected at the blood-brain and blood-cerebrospinal barriers, where it restricts xenobiotic penetration of the brain [16,105]. Zhuang et al. [106] have further shown that the oral administration of gefitinib increases topotecan penetration into the brain extracellular fluid but conversely decreases the ventricular cerebrospinal fluid penetration of topotecan. BCRP is also expressed in the intestinal epithelial cells, and Cusatis et al. [92] demonstrated that diarrhea, an adverse event related to oral gefitinib administration, is linked to genetic polymorphisms of BCRP, most notably C421A (Q141K). The resulting C421A variant of BCRP reduces its efflux activity [52] such that orally administrated gefitinib (presumably at submicromolar concentration *in vivo*) is also thought to inhibit BCRP function. Collectively, these observations strongly indicate that gefitinib at low concentrations (nM) is recognized as a substrate by BCRP, and that BCRP is one of the important mediators of gefitinib sensitivity both *in vitro* and *in vivo*.

Analogous to gefitinib, other small molecular kinase inhibitors of the HER-family members have been found to interact with BCRP. Erlotinib, a tyrosine kinase inhibitor with similarities to gefitinib, has been shown to interact with BCRP [95,107]. At relatively high concentrations, this drug shows antagonistic activity toward both BCRP and P-glycoprotein and can reverse the multidrug resistance by inhibiting the drug efflux activity of BCRP. In addition, BRCP expression was shown to decrease the intracellular accumulation of both erlotinib and gefitinib, and our unpublished findings indicate that lower concentrations of erlotinib are likely to be transported by BCRP. In studies of drug interactions *in vivo*, two polymorphic loci were identified in the BCRP promoter, −15622C/T and 1143C/T, which cause lower protein expression and are associated with improved pharmacokinetic parameters for erlotinib [96]. Hence, BCRP appears to recognize erlotinib as a substrate when this drug is administered at relatively low concentrations.

Canertinib also appears to be a substrate for BCRP since it has been shown to be transported by BCRP [94]. The intracellular accumulation of canertinib was shown to be reduced by the overexpression of BCRP and treatment with this agent was found to sensitize BCRP-expressing cells to SN-38 and topotecan via an increased intracellular accumulation of these drugs. Lapatinib is another newly developed kinase inhibitor that targets the HER-family that is a substrate and inhibitor of BCRP and P-glycoprotein [97]. *In vivo* studies show that the efflux transporters at the blood-brain barrier influence lapatinib penetration into the brain. However, BCRP appears to have little impact upon the intestinal absorption of lapatinib because systemic exposure to this drug by oral dosing was unchanged even when BCRP and P-glycoprotein are absent from the gastrointestinal tract.

4.3. Interactions between kinase inhibitors and BCRP

In addition to clinical kinase inhibitors, a variety of kinase inhibitory compounds appear to interact with ABC transporters [76,101,108–113]. Most of the recently developed protein kinase inhibitors are designed to compete with ATP binding to the kinase domain, thereby exerting their suppressive effects [114]. Typical of such molecules, imatinib and gefitinib also have inhibitory activity against ABC transporters that contain ATP-binding domains. Most BCRP-interactive kinase inhibitors used at relatively high concentrations were thus initially predicted to block the ATPase activity of this protein. However, Saito et al. [115] have demonstrated that gefitinib binds to ATP-bound BCRP, indicating that the as yet to be determined gefitinib-binding site in BCRP is not the ATP-binding domain. Photo affinity labeling using ^{125}I-labeled iodoarylazidoprazosin, a typical substrate for P-glycoprotein and BCRP, has been widely used for competition experiments with sample compounds at ABC transporter substrate-binding sites. Using this technique, Brendel et al. showed that imatinib and nilotinib bind to BCRP at the substrate-interaction site [86], but Shi et al. demonstrated that erlotinib has little ability to compete with iodoarylazidoprazosin at the substrate-binding sites of BCRP and P-glycoprotein [95]. In regard to a drug-BCRP interaction model, some interesting studies have proposed the presence of multiple drug binding sites on this ABC transporter [116,117] that may include kinase inhibitors. Further studies will be needed to properly elucidate the modes of interaction between kinase inhibitors and BCRP.

5. Other BCRP inhibitors

BCRP inhibitors may have important clinical applications as modulators of the efficacy of cancer drugs that are BCRP substrates. Co-administration of such inhibitors may overcome BCRP-mediated drug resistance in some tumor cells and will necessarily affect the pharmacokinetics and pharmacodynamics of BCRP-substrates in tissues. This may however have consequences in terms of the increased toxicity of specific anticancer agents. Various compounds, including Fumitremorgin C, have been found to reverse drug resistance through the inhibition of BCRP function [105]. The placenta synthesizes and secretes estrogens and BCRP is highly expressed in the syncytiotrophoblasts of the placenta [26]. Other than protein kinase inhibitors, we anticipated in our early work that estrogens would interact with BCRP as a physiological substrate or as an inhibitor. We have since found that estrone and 17βestradiol can restore drug sensitivity in *BCRP*-transduced human myelogenous leukemia K562 (K562/BCRP) cells [118]. In addition, we have also examined estrogen agonists, antagonists, and their derivatives as potential BCRP-reversing agents [12]. Although neither tamoxifen nor toremifene was found to have any effects upon topotecan uptake in K562/BCRP cells, diethylstilbestrol showed strong BCRP-reversing activity. Diethylstilbestrol enhances the cellular accumulation of topotecan and reverses the resistance to SN-38 and mitoxantrone in K562/BCRP cells without affecting the parental K562 cells. Further screening identified TAG-139, a derivative of tamoxifen, as a strong BCRP inhibitory agent. TAG-139 reversed both SN-38 and mitoxantrone-resistance in K562/BCRP cells with a 5-fold greater potency than estrone. Intriguingly, the dose-dependent characteristics of drug resistance reversal by TAG-139 and estrone appear to be similar, suggesting that tamoxifen derivatives and estrone may interact with the same binding site of BCRP.

Some phytoestrones and flavonoids have weak estrogenic activity, and we have shown in our laboratory that some of these compounds, including genistein, naringenin, acacetin, kaempferol and some glycosylated flavonoids, are effective BCRP-inhibitors and potentiate the cytotoxicity of SN-38 and mitoxantrone in K562/BCRP cells [119,120]. However, genistein and naringenin were unable to reverse either P-glycoprotein-mediated vincristine resistance or MRP1-mediated etoposide resistance. Our findings have indicated that genistein is a naturally occurring substrate of BCRP and competitively inhibits BCRP-mediated drug efflux.

We have developed a number of new inhibitors of BCRP in our laboratory, including a novel acrylonitrile derivative YHO13351 and its parent compound YHO13177. Both of these molecules enhance the *in vitro* cytotoxicity of SN-38 in the human lung cancer cell lines NCI-H460 and NCI-H23, the leukemia cell line RPMI8226, and the pancreatic cancer cell line AsPC-1. All of these cell lines express BCRP, and the effects of YHO13351 and YHO13177 were dose dependent. YHO13177 was found *in vitro* to reverse SN-38-, mitoxantrone-, and topotecan-resistance in *BCRP*-transduced HCT-116 cells, but it showed little effect upon P-glycoprotein-mediated paclitaxel resistance. Moreover, YHO-13351 markedly reduced HCT-116/BCRP tumor

growth in a xenograft model when combined with half of the maximal tolerated dose of CPT-11. These findings suggest that YHO-13351 may be a clinically useful drug that can reverse BCRP-mediated drug resistance to CPT-11, mitoxantrone, or topotecan.

6. Perspectives

A number of studies now strongly suggest that BCRP expression is associated with the clinical efficacy of a specific class of anticancer drugs. The accelerated development of anticancer drugs continues to produce a growing number of novel molecular targeted agents such as the small molecule protein kinase inhibitors [67,121]. Molecular analyses of the functional interactions between such novel drugs and the ABC transporter BCRP suggest their usefulness as indicators of the clinical efficacy of these anticancer agents in individual patients. In addition, the increased risk of adverse effects resulting from the use of putative BCRP substrates needs to be evaluated when considering combinations of protein kinase inhibitors, even at clinically relevant dosages.

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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;

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 microenvironmental 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

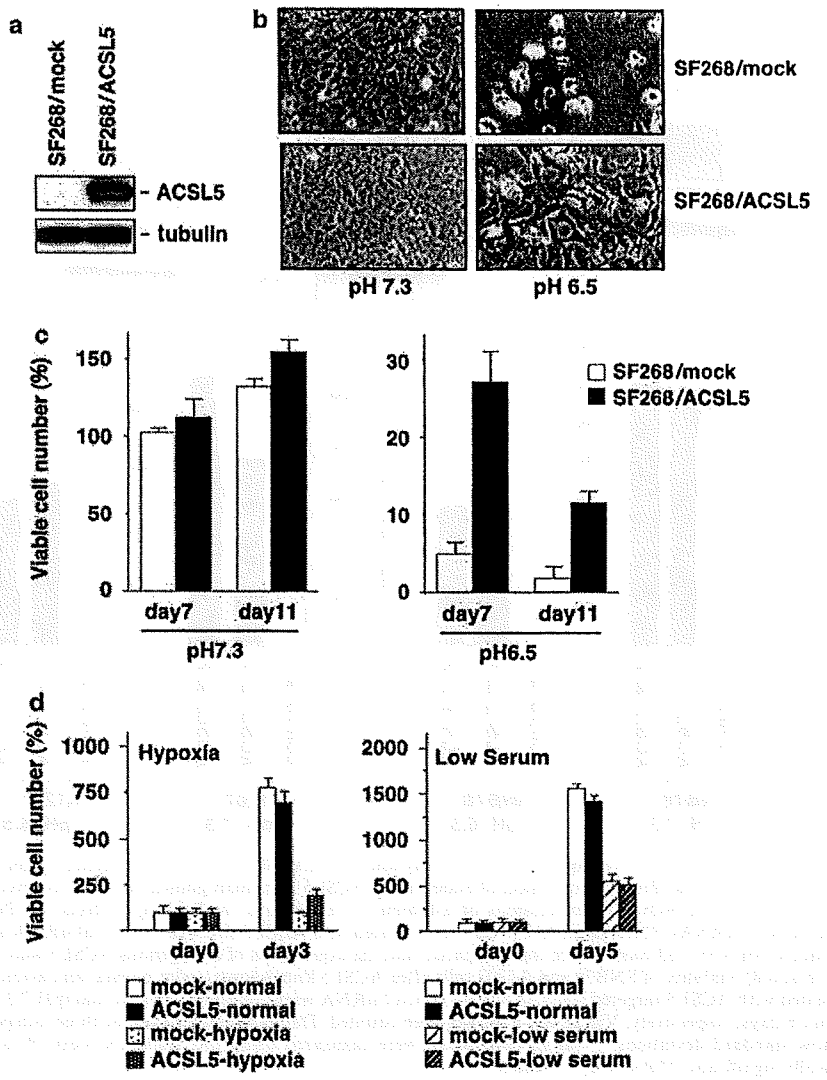

Figure 1 Acyl-CoA synthetase 5 (ACSL5) promotion of survival of human glioma SF268 cells under extracellular acidosis conditions. (a) The expression of FLAG epitope-tagged ACSL5 in transduced SF268 cells as revealed by western blot analysis with an anti-ACSL5 antibody. The expressions of α-tubulin were 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

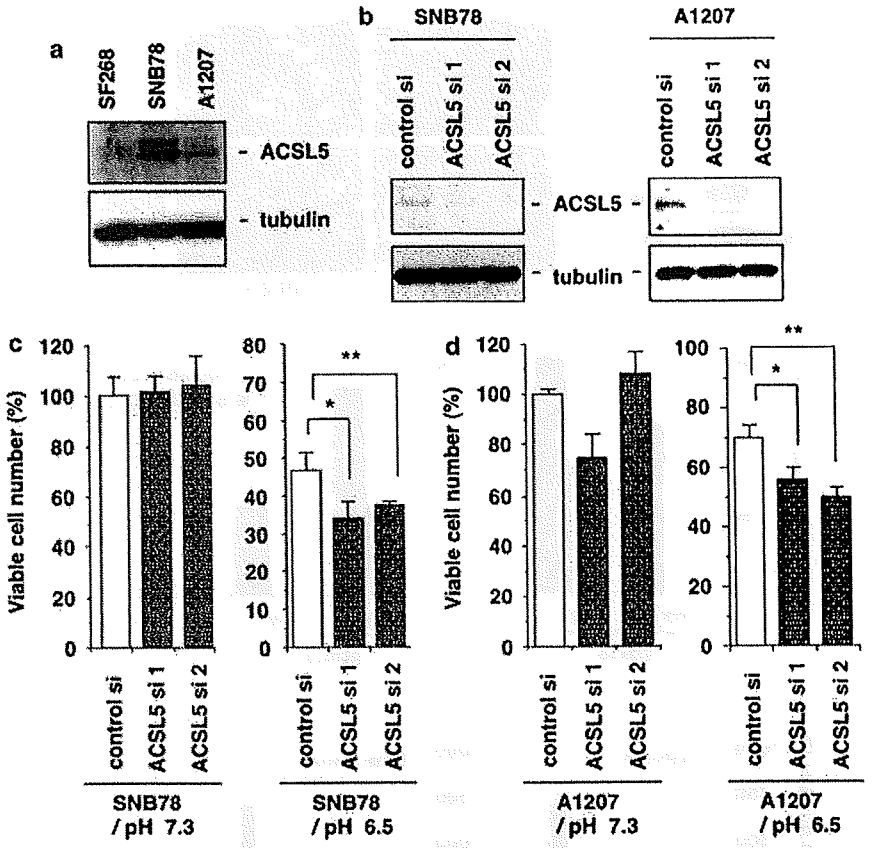

Figure 2 Involvement of endogenously overexpressed acyl-CoA synthetase 5 (ACSL5) in survival of human glioma SNB78 cells under extracellular acidosis conditions. (a) Protein expression of endogenous ACSL5 in human glioma cell lines as revealed by western blot analysis with an anti-ACSL5 antibody. The 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

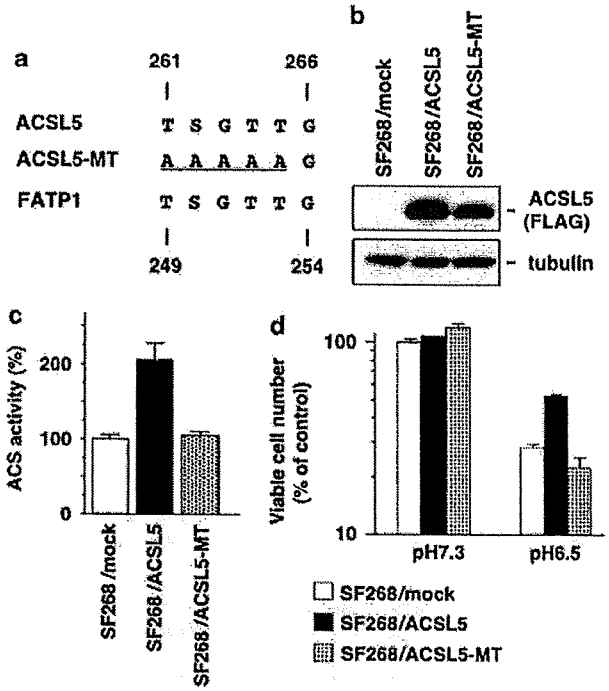

Figure 3 Acyl-CoA synthetase 5 (ACSL5) catalytic activity-dependent cell survival under extracellular acidosis conditions. (a) The amino-acid sequences of the putative active site in ACSL5 and FATP1. The amino-acid sequence, TSGTT (261–265), in wild-type ACSL5 was converted to AAAAA in ACSL5-MT. (b) 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
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	by ACSL5
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

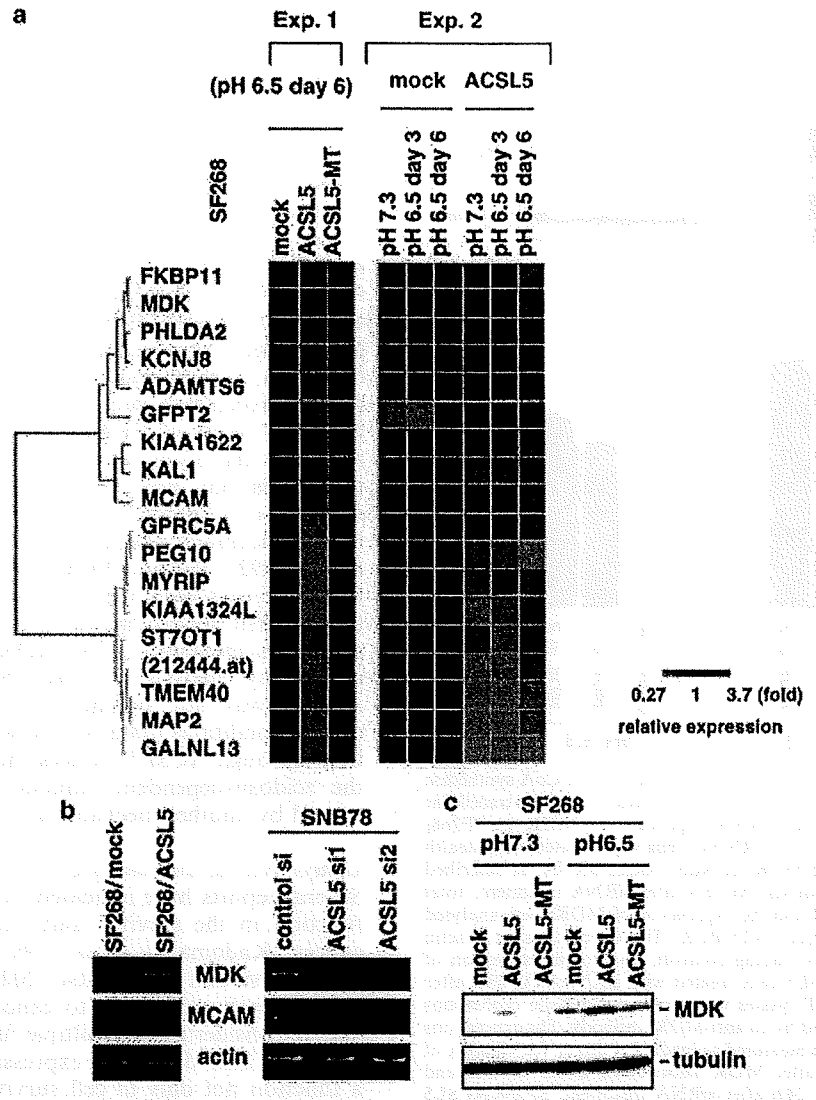

Figure 4 Identification of acyl-CoA synthetase 5 (ACSL5)-regulated gene expression signature by cDNA microarray analysis. (a) Hierarchical clustering using log-transformed relative expression changes over control for genes up- or downregulated exclusively in SF268/ACSL5 cells but not in SF268/ACSL5-MT 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

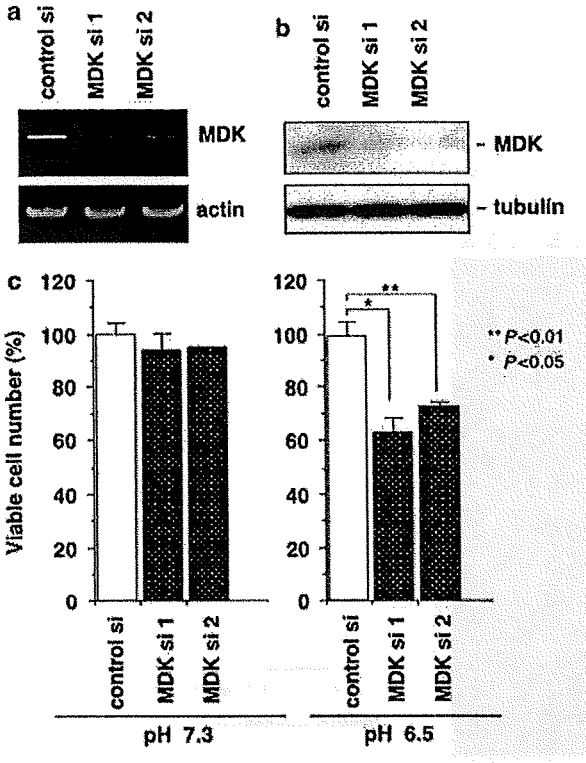

Figure 5 Involvement of midkine (MDK) in acyl-CoA synthetase 5 (ACSL5)-mediated glioma cell survival under extracellular acidosis conditions. (a) mRNA expression of MDK in SF268/ACSL5 cells treated with siRNAs. Cells were treated with stealth siRNAs that targeted MDK, or with control siRNA, as described 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 (~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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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)