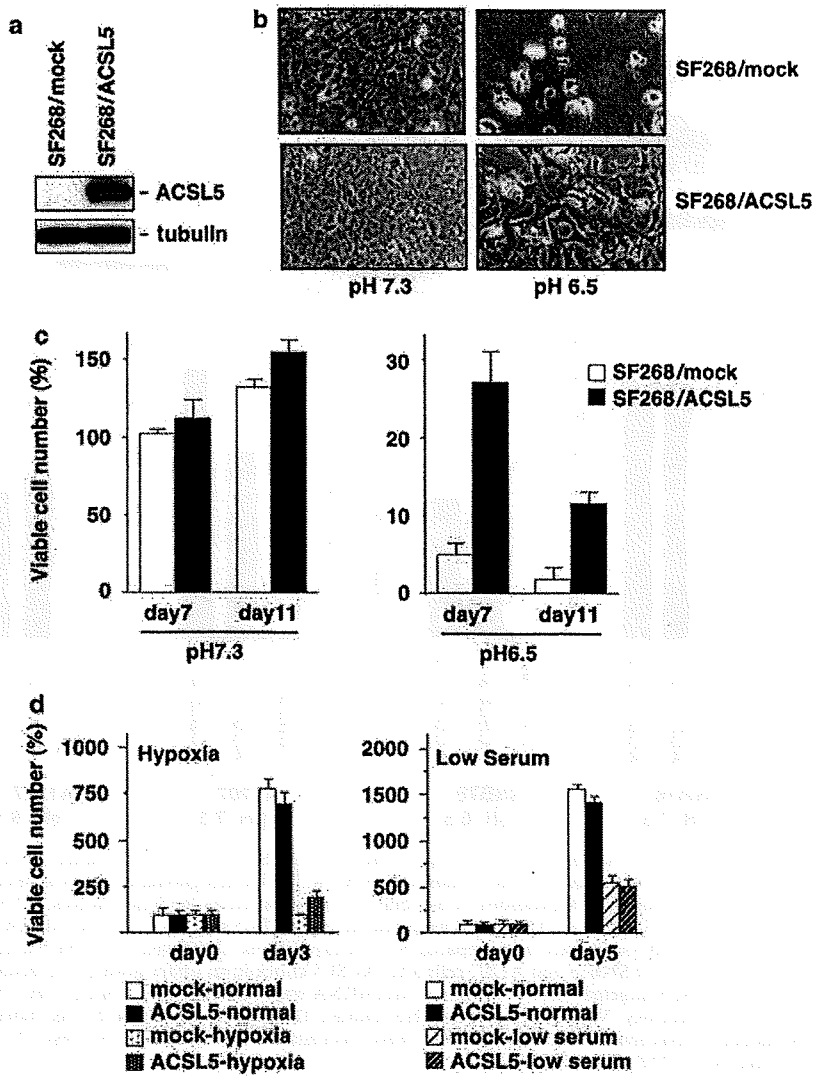

Figure 1 Acyl-CoA synthetase 5 (ACSL5) promotion of survival of human glioma SF268 cells under extracellular acidosis conditions. (**a**) The expression of FLAG epitope-tagged ACSL5 in transduced SF268 cells as revealed by western blot analysis with an anti-ACSL5 antibody. The expressions of α-tubulin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

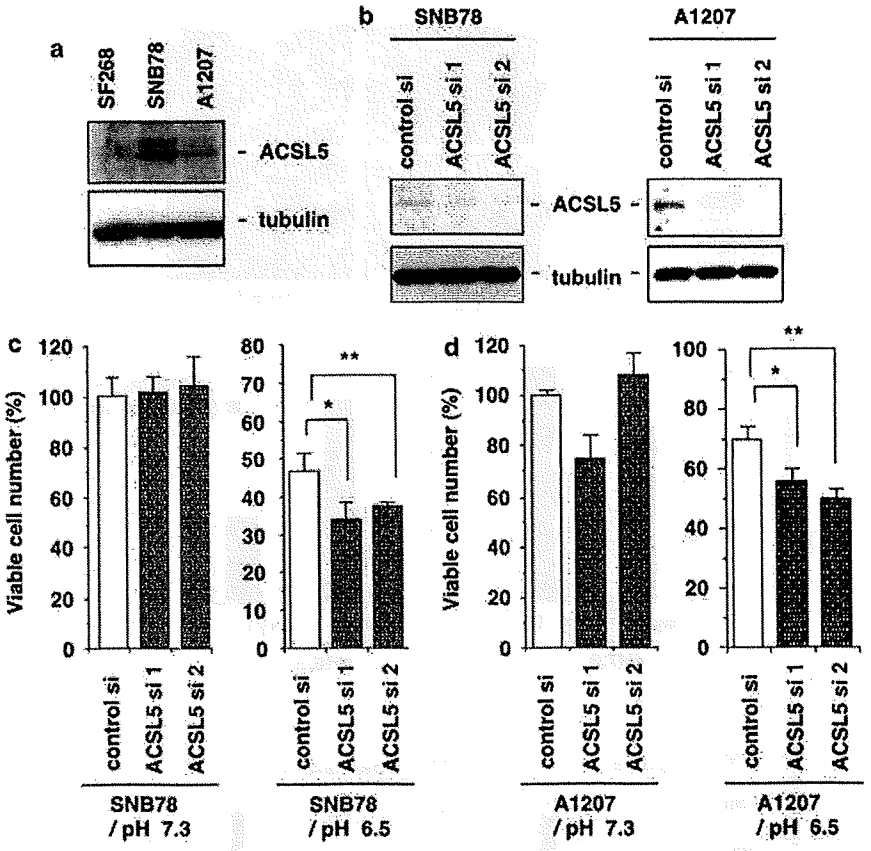

Figure 2 Involvement of endogenously overexpressed acyl-CoA synthetase 5 (ACSL5) in survival of human glioma SNB78 cells under extracellular acidosis conditions. (**a**) Protein expression of endogenous ACSL5 in human glioma cell lines as revealed by western blot analysis with an anti-ACSL5 antibody.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

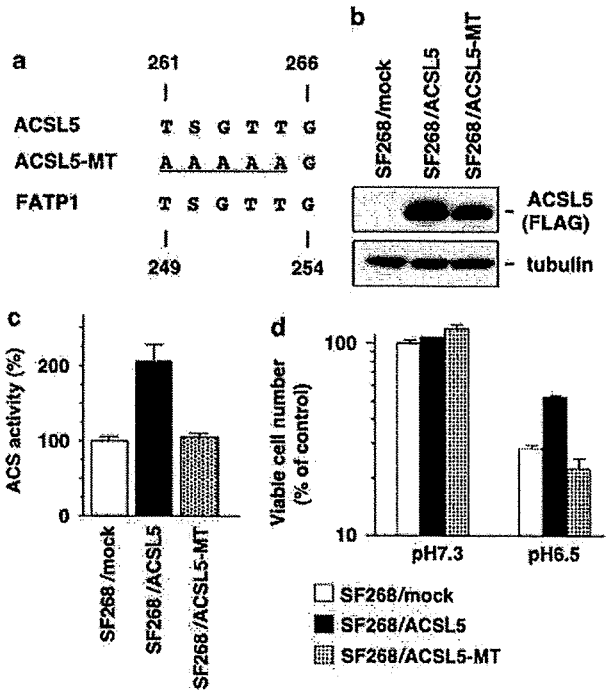

Figure 3 Acyl-CoA synthetase 5 (ACSL5) catalytic activity-dependent cell survival under extracellular acidosis conditions. (**a**) The amino-acid sequences of the putative active site in ACSL5 and FATP1. The amino-acid sequence, TSGTT (261–265), in wild-type ACSL5 was converted to AAAAA in ACSL5-MT.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

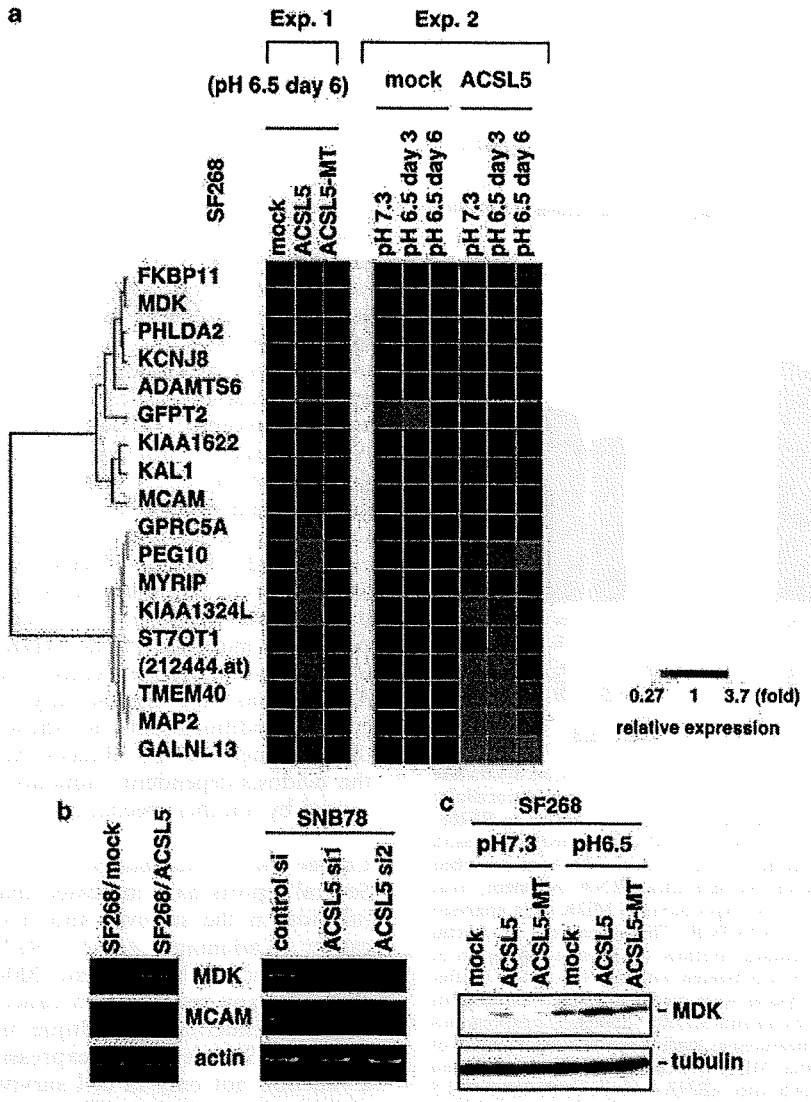

Figure 4 Identification of acyl-CoA synthetase 5 (ACSL5)-regulated gene expression signature by cDNA microarray analysis. (a) Hierarchical clustering using log-transformed relative expression changes over control for genes up- or downregulated exclusively in SF268/ACSL5 cells but not in SF268/ACSL5-MT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

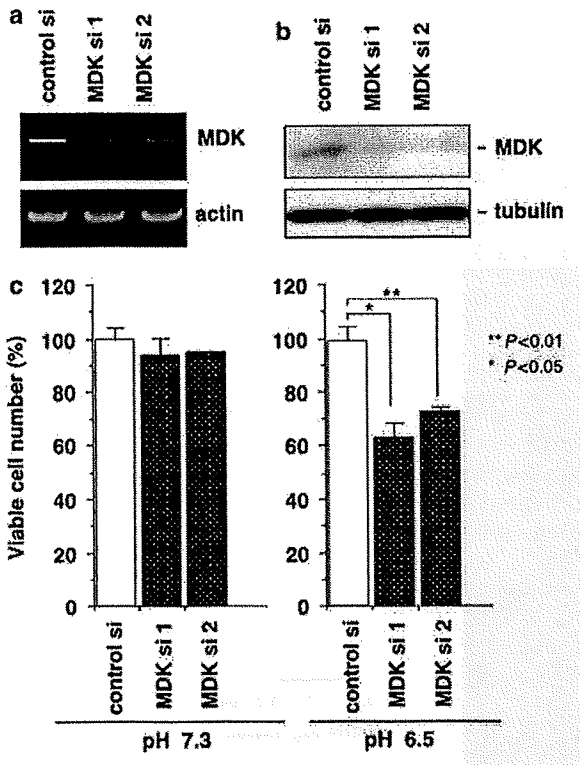

Figure 5 Involvement of midkine (MDK) in acyl-CoA synthetase 5 (ACSL5)-mediated glioma cell survival under extracellular acidosis conditions. (a) mRNA expression of MDK in SF268/ACSL5 cells treated with siRNAs. Cells were treated with stealth siRNAs that targeted MDK, or with control siRNA, as described 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 QuikChange 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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ORIGINAL ARTICLE

Flavonoids inhibit breast cancer resistance protein-mediated drug resistance: transporter specificity and structure–activity relationship

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Abstract
Purpose ATP-binding cassette (ABC) transporters, such as P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), and multidrug resistance-related protein 1 (MRP1), confer resistance to various anticancer agents. We previously reported that some flavonoids have BCRP-inhibitory activity. Here we show the reversal effects of an extensive panel of flavonoids upon BCRP-, P-gp-, and MRP1-mediated drug resistance.
Methods Reversal effects of flavonoids upon BCRP-, P-gp-, or MRP1-mediated drug resistance were examined in the *BCRP*- or *MDR1*-transduced human leukemia K562 cells or in the *MRP1*-transfected human epidermoid carcinoma KB-3-1 cells using cell growth inhibition assays. The IC_{50} values were determined from the growth inhibition curves. The RI_{50} values were then determined as the concentration of inhibitor that causes a twofold reduction of the IC_{50} in each transfectant. The reversal of BCRP activity was tested by measuring the fluorescence of intracellular topotecan.
Results The BCRP-inhibitory activity of 32 compounds was screened, and 20 were found to be active. Among these active compounds, 3′,4′,7-trimethoxyflavone showed the strongest anti-BCRP activity with RI_{50} values of 0.012 μM for SN-38 and 0.044 μM for mitoxantrone. We next examined the effects of a panel of 11 compounds on P-gp- and MRP1-mediated drug resistance. Two of the flavones, 3′,4′,7-trimethoxyflavone and acacetin, showed only low anti-P-gp activity, with the remainder displaying no suppressive effects against P-gp. None of the flavonoids that we tested inhibited MRP1.
Conclusion Our present results thus indicate that many flavonoids selectively inhibit BCRP only. Moreover, we examined the structure–BCRP inhibitory activity relationship from our current study.

Keywords BCRP/ABCG2 · P-glycoprotein/ABCB1 · MRP1/ABCC1 · Flavonoid · Growth inhibition assay

Abbreviations

ABC	ATP-binding cassette
BCRP	Breast cancer resistance protein
MDR	Multidrug resistance
P-gp	P-glycoprotein
MRP1	Multidrug resistance-related protein 1
SN-38	7-Ethyl-10-hydroxycamptothecin (the active metabolite of irinotecan)
VP-16	Etoposide

Introduction

Tumor cells often acquire multidrug resistance, characterized by cross-resistance to other structurally unrelated

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agents [1]. Multidrug-resistant cells express ABC transporters, such as the *MDR1* gene product P-glycoprotein (P-gp)/ABCB1, breast cancer resistance protein (BCRP)/ABCG2, and multidrug resistance-related protein 1 (MRP1)/ABCC1, that pump out various structurally unrelated anticancer agents in an ATP-dependent manner. In the 1980s, verapamil was firstly found to increase the intracellular concentration of anticancer agents in multidrug-resistant cells by binding P-gp and inhibiting the P-gp-mediated drug efflux [2, 3]. Subsequently, many P-gp inhibitors such as valspoder (PSC-833), dofequidar fumarate (MS-209), tariquidar (XR9576), and thiose-micarbazone derivative (NSC73306) have been developed that also interact with P-gp and reverse P-gp-mediated drug resistance [4–7]. Clinical trials using such P-gp inhibitors have shown an in vivo increase in the intracellular concentration of coadministered anticancer agents in P-gp-positive tumor cells [8]. However, phase III trials of these agents have not been successful and no significant survival benefit of P-gp inhibition has yet been achieved [9, 10]. Further clinical studies using new P-gp inhibitors and new combination treatment regimens have been devised however, and some are now ongoing.

BCRP is a half-molecule ABC transporter with an NH_2-terminal ATP-binding site and COOH-terminal transmembrane domain [11–15]. Recently, we reported that BCRP forms homodimers via a disulfide bridge between Cys603, a residue on the third outer-membrane domain of the BCRP monomer [16, 17]. The homodimeric BCRP complex acts as an efflux pump for various anticancer agents including SN-38, mitoxantorone, and topotecan, and thus prevents the build up of high intracellular concentrations of such anticancer agents and decreases their cytotoxic effects. BCRP is reportedly also expressed in various normal human tissues and cells, such as the placenta, liver, brain, spinal code, adrenal gland, testes, prostate, uterus, kidney, heart, bone marrow, and small intestine [18]. Furthermore, BCRP is expressed in hematopoietic stem cells and is thought to be a stem cell marker [19]. We previously reported that estrone and 17β-estradiol inhibit BCRP-mediated drug transport and resistance. In addition, we have found from our studies that BCRP transports sulfated estrogens as physiologic substrates but not as free estrogens [20, 21]. We further demonstrated that some flavonoids, such as genistein and naringenin, diminished the function of BCRP as an efflux pump and reversed BCRP-mediated resistance to anticancer agents [22]. Flavopiridol, a flavonoid-derived antitumor agent, is a substrate of BCRP [23], and flavonoids and estrogenic compounds thus possess BCRP inhibitory properties.

In our current study, we screened a further panel of flavonoids possessing inhibitory activity for BCRP, including 29 flavonoids and 3 flavonoid-related compounds (total 32), by cell growth inhibition assay. We find that 20 of these compounds harbor inhibitory activity against BCRP. However, although two of the flavonoids that we tested induced a weak reversal of P-gp-mediated multidrug resistance, none of the other compounds displayed any inhibitory properties toward P-gp. Additionally, none of the flavonoids screened in this study were found to inhibit MRP1. We thus conclude that they selectively target BCRP only.

Materials and methods

Reagents

Flavonoid compounds were purchased from Funakoshi (Tokyo, Japan). Fumitremorgin C (FTC) was purchased from Alexis (San Diego, CA, USA). Anti-BCRP polyclonal antibody (3488) was raised by immunizing rabbits with a KLH-conjugated 20-mer peptide corresponding to the amino acid region 340–359 of the human BCRP protein [16]. The anti-P-gp monoclonal antibody (C219) was purchased from Zymed (South San Francisco, CA, USA), and the anti-MRP1 monoclonal antibody (MRPm6) was obtained from Nichirei (Tokyo, Japan).

Cells and cell culture

K562/BCRP and K562/MDR cells were established from human leukemia K562 cells in our laboratory [22], and grown in RPMI 1640 medium supplemented with 7% fetal bovine serum at 37°C in 5% CO_2. Human epidermoid carcinoma KB-3-1 cells were cultured in DMEM supplemented with 7% fetal bovine serum at 37°C in 5% CO_2. KB/MRP1 cells were established using the following procedures: KB-3-1 cells were transfected with the pCAL-MRP1 construct bearing the human *MRP1* cDNA insert by the use of the Mammalian Transfection Kit (Stratagene, La Jolla, CA, USA). This was followed by selection with increasing concentrations of etoposide (VP-16). The cells were subcloned, and the MRP1 expression levels of each clone was confirmed by western blotting with an anti-MRP1 monoclonal antibody. The western blotting procedure used has been described previously [16]. Subclone 14 showed the highest expression of MRP1 and these cells were thus further selected with increasing concentrations of doxorubicin for 4 weeks and designated as KB/MRP1 cells.

Growth inhibition assay

The effects of flavonoids on the sensitivity of cells to various cytotoxic agents were evaluated by measuring cell growth inhibition after incubation at 37°C for 5 days in the absence or presence of various concentrations of anticancer drugs in combination with the test compounds. Cell numbers were determined with a Coulter counter. The IC_{50} values (drug dose causing 50% inhibition of cell growth) were determined from the growth inhibition curves. The RI_{50} values were then determined as the concentration of inhibitor that causes a twofold reduction of the IC_{50} in each transfectant. RI_{50}^{-1}, the reciprocal value of RI_{50}, was also used as a reverse activity measurement of drug resistance.

Topotecan uptake

The intracellular accumulations of topotecan were determined by measuring the fluorescence spectrophotometrically. K562 or K562/BCRP cells (2×10^6 cells) were suspended in 1 ml of RPMI 1640 medium containing 0.5 μM topotecan and appropriate concentrations of the compounds. The cells were incubated at 37°C for 30 min, and washed with ice-cold PBS. The intracellular topotecan was extracted from the cells with 1 ml of ethanol. The intensity of topotecan fluorescence was measured using a fluorescent spectrophotometer.

Results

To screen the various flavonoids under analysis, we used *MDR1*, *BCRP*, and *MRP1* cDNA transfectants. The expression of the ABC transporters in each transfectant was first confirmed by western blot. The parental cells, K562 or KB-3-1, did not express any of the three ABC transporters, whereas the K562/BCRP, K562/MDR, and KB/MRP1 cells expressed exogenous BCRP, P-gp, and MRP1, respectively (Fig. 1). These transfectants did not express any of other transporters (Fig. 1). We next examined the degree of resistance to various anticancer agents in the K562/BCRP, K562/MDR, and KB/MRP1 cells, compared with the corresponding parental cells. K562/BCRP cells showed a 21-fold higher resistance to SN-38 than K562 cells (Table 1). K562/MDR cells showed 160-fold higher resistance to vincristine than K562 cells (Table 1). In addition, the KB/MRP1 cells that we established in this study showed resistance to VP-16, doxorubicin, vincristine, and SN-38 (Table 1), which is similar to the cross-resistant patterns in other MRP-expressing cells [23–25].

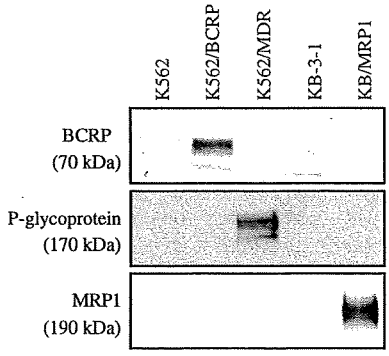


Fig. 1 Analysis of the expression levels of BCRP, P-gp, and MRP1 in stably transfected cells by western blot. Cell lysates (20 μg/lane) were resolved by SDS-PAGE and transferred onto nitrocellulose membranes. The expression levels of BCRP, P-gp, or MRP1 were then detected by incubation of the membrane with anti-BCRP polyclonal antibody (3488), anti-P-gp monoclonal antibody (C219), or anti-MRP1 monoclonal antibody (MRPm6), respectively

Table 1 Drug resistance of each resistant cells

Resistant protein	Resistant cells	Relative resistance factor			
		SN-38	VCR	Dox	VP-16
BCRP	K562/BCRP	21	ND	ND	ND
P-gp	K562/MDR	ND	160	ND	ND
MRP1	KB/MRP	2.6	25	8.8	12

Parental or resistant cells were cultured for 5 days with increasing concentrations of the indicated drugs. Cell numbers were counted with a Coulter counter, and IC_{50} was determined. Relative resistance factor is the ratio of IC_{50} for the resistant cells divided by that for the parental cells

VCR Vincristine, *Dox* doxorubicin, ND not determined

The structures, symbols, and compound names of the flavonoids and their related agents used in this study are shown in Fig. 2. Each compound itself showed no or only marginal growth inhibitory effect on the cells in the concentrations used in this study (data not shown). We examined the effects of these compounds on SN-38 and mitoxantrone (MXR) resistances in K562/BCRP cells (Figs. 3, 4). The results shown in Fig. 3a indicate that 3′,4′,7-trimethoxyflavone (Fig. 2; 1-a) strongly suppressed BCRP-mediated multidrug resistance. This is evident from the growth inhibition curves of the K562/BCRP cells treated with this flavonoid at low concentrations (0.03 and 0.1 μM; closed triangle and lozenge, respectively) as they are well shifted to the left compared with the untreated cells (closed circle). The growth inhibition curve of K562/BCRP cells treated with 1 μM apigenin (Fig. 2;

A

1. Flavone	5	7	3'	4'	
1-a		OCH_3	OCH_3	OCH_3	3',4',7-trimethoxyflavone
1-b	OH	OH		OCH_3	Acacetin
1-c	OH	OH	OH	OCH_3	Diosmetin
1-d	OH	OH		OH	Apigenin
1-e	OH	OH			Chrysin
1-f	OH	OH	OH	OH	Luteolin
1-g	OH	OH	OH	G_3	Luteolin-4'-O-glucoside
1-h	OH	G_1			Rhoifolin
1-i	OH	G_2	OH	OCH_3	Diosmin

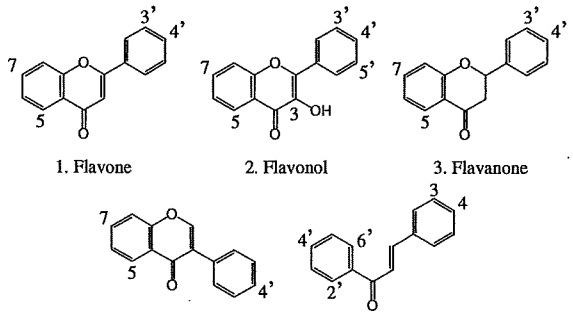
2. Flavonol	3	5	7	3'	4'	5'	
2-a		OH	OH		OCH_3		Kaempferide
2-b		OH	OH		OH		Kaempferol
2-c		OH	OH				Galangin
2-d		OH	OH	OH	OH	OH	Myricetin
2-e		OH	OH	OH	OH		Quercetin
2-f	G_3	OH	OH		OH		Kaempferol-3-O-glucoside
2-g		OH	G_1		OH		Kaempferol-7-O-nephesperidose
2-h			OH	OH	OH		Fisetin
2-i	G_4	OH	OH	OH	OH		Peltatoside
2-j		OH	OH	OCH_3	OH		Rutin

3. Flavanone	5	7	3'	4'	
3-a	OH	OH	OH	OCH_3	Hesperetin
3-b	OH	G_1		OH	Naringenin-7-O-glucoside
3-c	OH	OH		OH	Naringenin
3-d	OH	OH	OH	OH	Eriodictyol

4. Isoflavone	5	7	4'	
4-a	OH	OH	OH	Genistein
4-b		OH	OH	Daidzein

5. Chalcone	3	4	2'	4'	6'	
5-a	OCH_3	OCH_3				3,4-dimethoxychalcone
5-b		OH	OH	OH	OH	Phloretin

B



C

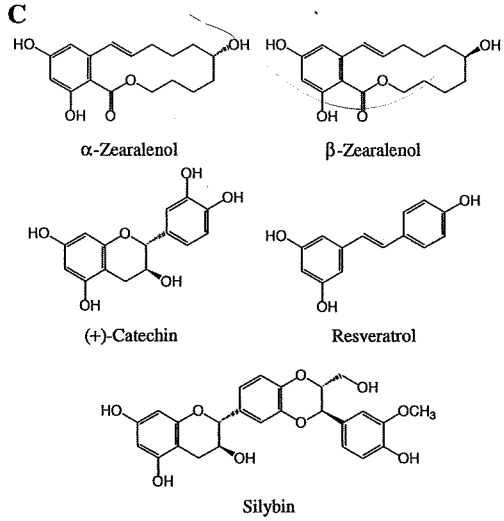


Fig. 2 Chemical structures of the flavonoids tested in the present study. **a** The structures of the flavones (9 compounds), flavonols (10 compounds), flavanones (4 compounds), isoflavones (2 compounds), and chalcones (2 compounds) screened in the present study. G_1, *O*-neohesperidoside; G_2, *O*-rutinoside; G_3, *O*-glucoside; G_4, *O*-arabinoglucoside. **b** Core structures of the five groups of compounds screened in the present study. **c** The flavonoid-related agents tested in the present study

1-d) (closed upward triangle) was also found to have shifted to the left compared with cells treated with 0.3 μM apigenin (closed downward triangle) or untreated (closed circle). This indicated that apigenin inhibits BCRP-mediated resistance to SN-38 and MXR (Fig. 3b). In contrast, diosmin (Fig. 2; 1-i) did not suppress BCRP-mediated drug resistance at any concentration (Fig. 3c). The reversal indices (RI_{50}) for SN-38 of 3′,4′,7-trimethoxyflavone, apigenin, and diosmin were measured as 0.012, 0.39, and >3 μM, respectively, and those for MXR were measured as 0.044, 0.62, and >3 μM, respectively. Other compounds were also examined using identical analyses, and the RI_{50} values for SN-38 were obtained from each growth inhibition curve. The RI_{50}^{-1} of the total panel of 32 compounds that we screened in this study are presented in Fig. 4, which shows that 20 of these 32 compounds can reverse BCRP-mediated SN-38 resistance. The lack of any reversal properties of the remaining 12 compounds was confirmed by treatments at the highest concentrations used in these experiments (data not shown). We also examined the reversal effects of a well-known BCRP inhibitor, FTC, as a positive control on BCRP-mediated drug resistances (Fig. 3d). As expected, FTC suppressed these resistances at the concentrations of 0.3 and 1 μM. The RI_{50} of FTC for SN-38 and that for MXR were measured as 0.24 and 0.23 μM, respectively.

We then examined the effects of the compounds on the BCRP-mediated efflux of topotecan. The intracellular topotecan in K562/BCRP cells was threefold lower than that in K562 cells (Fig. 5). 3′,4′,7-Trimethoxyflavone

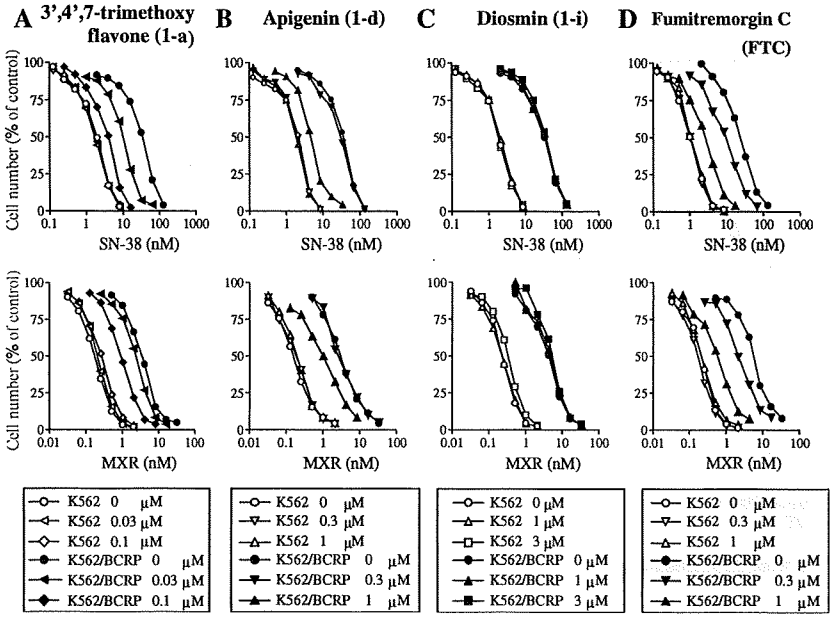


Fig. 3 Reversal effects of flavonoids on BCRP-mediated anticancer drug resistance. K562 (*open symbols*) and K562/BCRP (*closed symbols*) cells were cultured for 5 days in the absence (*circle*) or presence of 0.03 μM (*leftward triangle*), 0.1 μM (*lozenge*), 0.3 μM (*downward triangle*), 1 μM (*upward triangle*), or 3 μM (*square*) of the indicated compounds under increasing concentrations of SN-38. Cell numbers were determined with using a Coulter counter. **a** Effects of 3',4',7-trimethoxyflavone (*1-a*) on the sensitivity to SN-38 in K562 and K562/BCRP cells. **b** Effects of Apigenin (*1-d*) on the sensitivity to SN-38 in K562 and K562/BCRP cells. **c** Effects of Diosmin (*1-i*) on the sensitivity to SN-38 in K562 and K562/BCRP cells. **d** Effects of Fumitremorgin C on the sensitivity to SN-38 in K562 and K562/BCRP cells. Data points are the measurements of the mean ± SD from triplicate determinations

(1-a) at 1 μM increased the intracellular topotecan in K562/BCRP cells to a similar level as that in K562 cells (Fig. 5). Apigenin (1-d) at 10 μM also increased the intracellular accumulation of topotecan in K562/BCRP cells (Fig. 5). Diosmin (1-i) treatment did not alter the intracellular levels of topotecan (Fig. 5). These results clearly indicate that 3',4',7-trimethoxyflavone (1-a) and apigenin (1-d) increase the intracellular concentrations of BCRP substrate anticancer agents, but diosmin (1-i) does not.

We next examined whether any of the compounds showing reversal effects against BCRP-mediated drug resistance showed any cross-reactivity against other ABC transporter-mediated drug resistance pathways. As shown in Fig. 6a and b (left panels), 3',4',7-trimethoxyflavone (1-a) weakly suppresses P-gp- but not MRP1-mediated drug resistance. In addition, apigenin (1-d) and diosmin (1-i) do not reverse either P-gp- or MRP1-mediated drug resistance (Fig. 6a, b, middle and right panels). Among the representative 11 compounds that we chose to analyze in this experiment from the 32 compound panel, only two (1-a and -b) in fact inhibited P-gp-mediated drug resistance and none suppressed MRP1-mediated drug resistance (Table 2). These compounds also did not show growth inhibitory effects against K562 and KB-3-1 cells at the highest concentrations used in these experiments (data not shown). These data indicate that many flavonoids are select inhibitors of BCRP only.

Discussion

Estrogens such as estrone and 17β-estradiol have been found to contain inhibitory activity against the BCRP-mediated multidrug resistance pathways [20]. In addition, sulfated estrogens are found to be physiological substrates of BCRP, suggesting that they compete with anticancer agents on efflux from cells [21]. Synthesized estrogen antagonists and agonists have also been demonstrated to reverse drug resistance and have structural similarities that can directly inhibit BCRP function and/or reduce its expression levels [26]. It is noteworthy also that some flavonoids have structures that somewhat resemble the estrogens and display weak estrogenic activity [27]. Significantly, we and others have now shown that flavonoids possess anti-BCRP activity [22, 28–31].

Some flavonoids have been reported to interact with and competitively inhibit ABC transporters, including P-gp, MRP1, MRP2, and cystic fibrosis transmembrane conductance regulator [32–37]. In this regard,

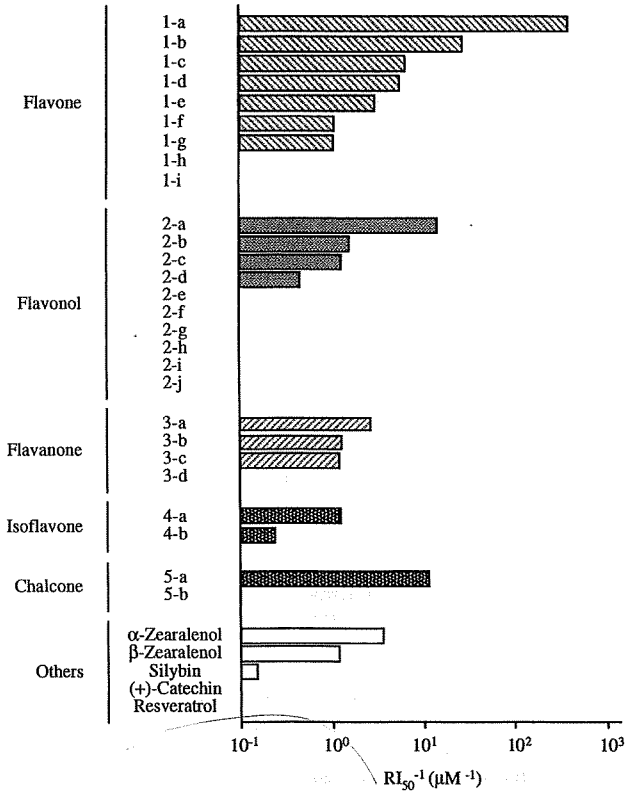


Fig. 4 Inhibitory effects of flavonoids on BCRP-mediated SN-38 resistance. K562 and K562/BCRP cells were cultured for 5 days in the absence or presence of the appropriate concentrations of flavonoids with increasing concentrations of SN-38. Cell numbers were determined with a Coulter counter. RI_{50} values were obtained graphically from the IC_{50} values of the K562 and K562/BCRP cells as described in Materials and methods. RI_{50}^{-1}, the reciprocal value of RI_{50}, was used to show the extent of drug resistance reversal of the compounds. These data are averaged from triplicate experiments

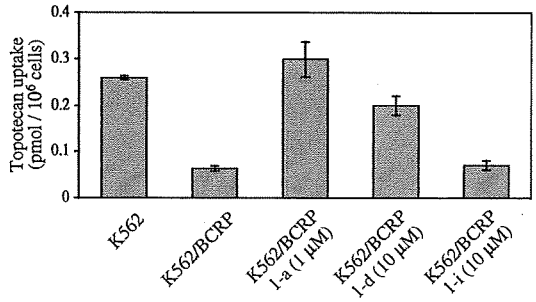


Fig. 5 Effect of flavonoids on topotecan uptake in K562/BCRP cells. K562 and K562/BCRP cells were incubated with 0.5 μM topotecan in the absence or presence of flavonoids at indicated concentrations at 37°C for 30 min. The intracellular topotecan was extracted from the cells with 1 ml of ethanol. The fluorescence intensity of topotecan was measured using a fluorescence spectrophotometer. Each *vertical bar* represents the mean ± SD of the measurements from triplicate determinations

genistein, naringenin, acacetin, kaempferol, quercetin, and flavopiridol have shown reversal effects against BCRP-mediated drug resistance in the previous studies from our laboratory and from other groups [22, 28, 38]. We therefore set out to screen additional flavonoids that may also reverse the effects of BCRP-mediated multidrug resistance in our present study. Of the 32 compounds that we tested, including the above flavonoids except flavopiridol, 20 compounds showed reversal effect for the resistance (Fig. 4). Of interest is that 3′,4′,7-trimethoxyflavone (1-a) has stronger inhibitory activity against BCRP than acacetin (1-b) that has been the strongest BCRP inhibitor in the flavonoids demonstrated in the previous study (Fig. 4). Almost all of the flavones, including 3′,4′,7-trimethoxyflavone, acacetin, diosmin, apigenin, chrysin, luteolin, luteolin-4′-*O*-glucoside, show BCRP-reversing activity (Figs. 3, 4). Moreover, many of the flavones, isoflavones, and chalcones tested in this study also reverse BCRP-mediated drug resistance, but 50% of the flavonols examined had no impact upon BCRP (Fig. 4).

From our present results, we propose a structure–activity relationship for BCRP inhibition by flavonoids (Fig. 7). We postulate that: (a) The double bond between position 2 and 3 of the C-ring is associated with high inhibitory activity against BCRP (Fig. 7a). As an example of this, apigenin (1-b) shows stronger suppressive activity toward BCRP than naringenin (3-c), and luteolin (1-f) is also more potent in this regard than eriodictyol (3-d) (Figs. 2, 4). (b) The 4′-*O*-methoxylation of the B-ring or the 4′-hydroxylation of the B-ring is also associated with more potent BCRP inhibition (Fig. 7b, c). In the former instance, hesperetin (3-a) has stronger BCRP inhibitory activity than eriodictyol (3-d), diosmetin (1-c) is more strongly inhibitory than luteolin (1-f), acacetin (1-b) is more potent than apigenin (1-d), and kaempferide (2-a) is a better inhibitor of BCRP than kaempferol (2-b) (Figs. 2, 4). In the case of 4′-hydroxylation of the B-ring, apigenin (1-d) showed slightly stronger BCRP suppression than chrysin (1-e), and kaempferol (2-b) is marginally more potent than galangin (2-c) (Figs. 2, 4). (c) The 3-hydroxylation of the C-ring or 3′-hydroxylation of the B-ring reduce BCRP inhibitory activity (Fig. 7d, e). In the former case, we mentioned our present results with galangin (2-c) compared with chrysin (1-e), kaempferol (2-b) compared with apigenin (1-d), kaempferide (2-a) compared with acacetin (1-b), and quercetin (2-e) compared with luteolin (1-f) (Figs. 2, 4). In the latter instance, our available examples include, luteolin (1-f) versus apigenin (1-d), eriodictyol (3-d) versus naringenin (3-c), and quercetin (2-e) versus kaempferol (2-b) (Figs. 2, 4). It is noteworthy also that in our present study, some glycosilated flavonoids showed anti-BCRP

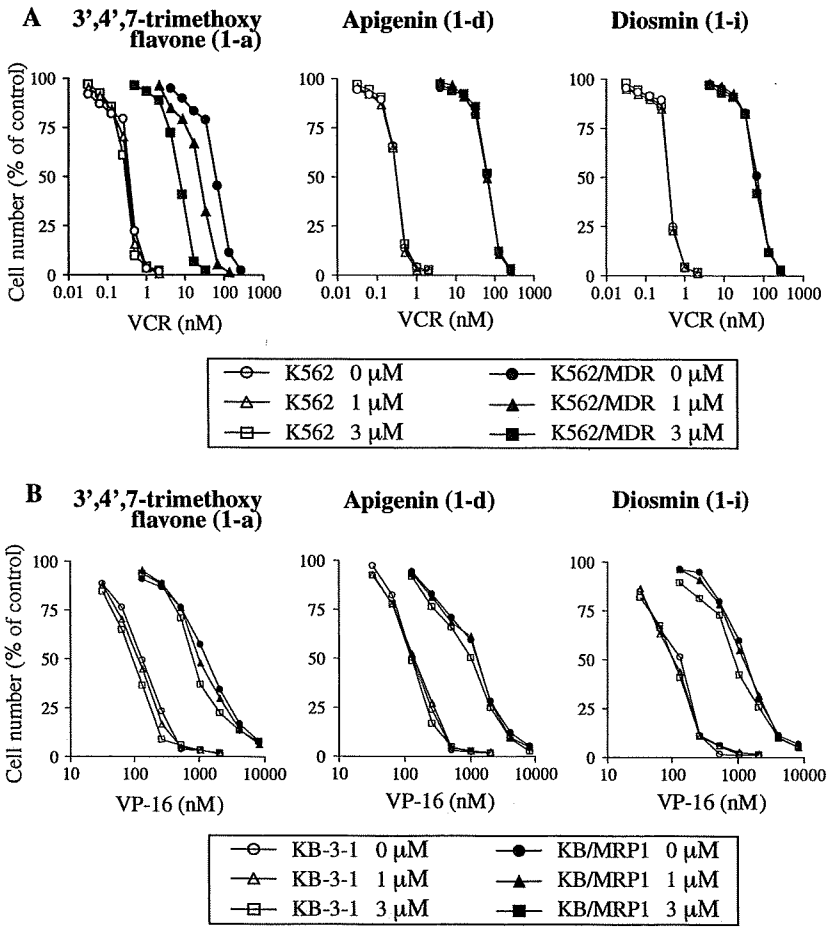


Fig. 6 Reversal effects of flavonoids on P-gp- or MRP1-mediated anticancer drug resistance. Parental cells (*open symbols*) and transfected cells (*closed symbols*) were cultured for 5 days in the absence (*circle*) or presence of 1 μM (*triangle*), or 3 μM (*square*) of the specific compounds indicated under increasing concentrations of anticancer agents. Cell numbers were determined using a Coulter counter. **a** Effects of flavonoid treatment upon the sensitivity to vincristine (VCR) in K562 and K562/MDR cells. **b** Effects of flavonoid treatment on the sensitivity to VP-16 in KB-3-1 and KB/MRP1 cells. Data points are measurements of the mean ± SD from triplicate determinations

Table 2 Reversal of BCRP-, P-gp-, and/or MRP1-mediated anticancer drug resistance by flavonoids

	RI_{50} (μM)		
	BCRP	P-gp	MRP1
1. Flavone			
1-a	0.012	0.68	>3
1-b	0.11	1.5	>3
1-d	0.39	>3	>3
1-e	0.67	>3	>3
1-f	1.5	>3	>3
1-h	>3	>3	>3
1-i	>3	>3	>3
2. Flavonol			
2-a	0.17	>3	>3
2-b	1.1	>3	>3
2-g	>3	>3	>3
2-h	>3	>3	>3

Parental or resistant cells were cultured for 5 days with increasing concentrations of anticancer drugs together with or without flavonoids. Cell numbers were counted with a Coulter counter, and RI_{50} was determined

activity. Glycosilated flavonoids may be useful for clinical practice because they are soluble in water and we would predict that both they and/or water-soluble derivatives of flavonoids will be developed as BCRP inhibitors in the future.

Flavonoids are safe nutrients, being the most abundant polyphenolic compounds present in the human diet in fruits, vegetables, and plant-derived beverages. It has been reported that a human adult normally assimilates 200–300 mg of flavonoids per day in their diet [39]. For example, 100 g of soybean contains 100–200 mg of isoflavones comprising genistein, daidzein, glycitein, and their corresponding glycosides. In the case of an intake of 50 mg of genistein, the peak plasma concentration of this compound was reported to reach a level of approximately 1 μM in healthy premenopausal women [40]. Therefore, flavonoids contained in foods can be considered to have a positive effect on the pharmacokinetics of anticancer agents. Hence, dietary controls will be necessary for patients undergoing cancer chemotherapy and it will also be important that the peak plasma concentrations of anticancer agents are continually monitored in these individuals.

In conclusion, we have shown that flavonoids selectively reverse BCRP-mediated drug resistance, that these compounds may be useful as BCRP inhibitors,

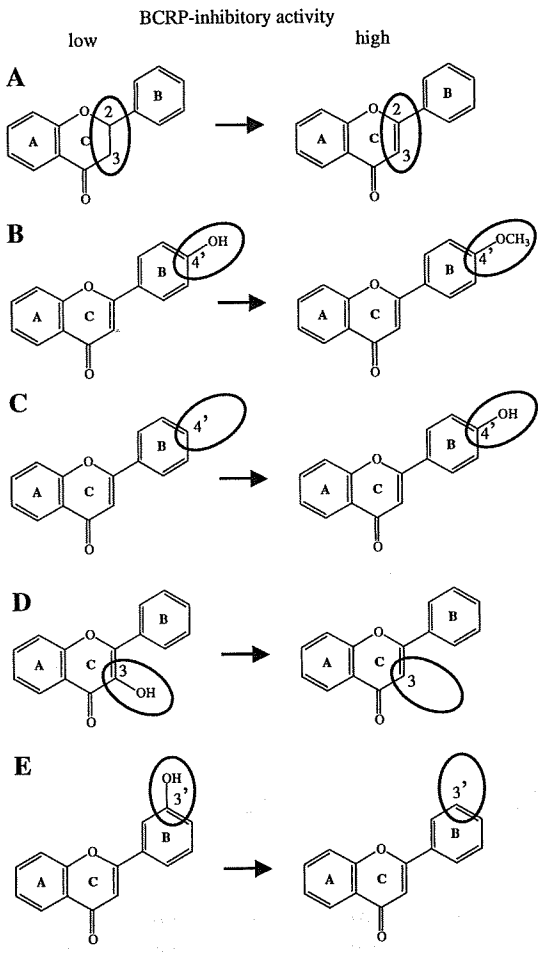


Fig. 7 Structure–activity relationship of BCRP inhibition by flavonoids

and that they are likely to bring clinical benefits via more effective and safer cancer chemotherapy treatments.

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Pilot study of *MDR1* gene transfer into hematopoietic stem cells and chemoprotection in metastatic breast cancer patients

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A major problem in high-dose chemotherapy with autologous hematopoietic stem cell transplantation is insufficient function of reconstituted bone marrow that limits the efficacy of post-transplantation chemotherapy. Because transduction of hematopoietic stem cells with the multidrug resistance 1 (*MDR1*) gene might circumvent this problem, we conducted a pilot study of *MDR1* gene therapy against metastatic breast cancer. Peripheral blood stem cells were harvested, and one-third of the cells were transduced with *MDR1* retrovirus. After the reconstitution of bone marrow function, the patients received high-dose chemotherapy with transplantation of both *MDR1*-transduced and unprocessed peripheral blood stem cells. The patients then received docetaxel chemotherapy. Two patients received transplantation of the *MDR1*-transduced cells in 2001. Peripheral blood *MDR1*-transduced leukocytes were 3–5% of the total cells after transplantation, but decreased gradually. During docetaxel chemotherapy, an increase in the rate of *MDR1*-transduced leukocytes (up to 10%) was observed. Comparison of docetaxel-induced granulocytopenia in the two patients suggested a bone marrow-protective effect of the *MDR1*-transduced cells. No serious side-effect was observed, and the patients were in complete remission for more than 3 years. The *MDR1*-transduced cells gradually decreased and disappeared almost entirely by the end of 2004. Results of linear amplification-mediated polymerase chain reaction of the *MDR1*-transduced leukocytes suggested no sign of abnormal amplification of the transduced cells. A third patient received transplantation of the *MDR1*-transduced cells in 2004. These results suggest the feasibility of our *MDR1* gene therapy against metastatic breast cancer, and follow-up is ongoing. (*Cancer Sci* 2007; 98: 1609–1616)

Although breast cancer is sensitive to chemotherapy and endocrine therapy, the prognosis of advanced or relapsed breast cancer is not satisfactory. The response rates of advanced breast cancers to most combination chemotherapies are between 40 and 70%, with complete response (CR) rates being 10–30%. The duration of response is 9–18 months for CR and 7–10 months for partial response (PR). Rates of long-term survivors, of more than 10 years, are less than 5%.[1,2]

High-dose chemotherapy (HDCT) with autologous hematopoietic stem cell transplantation (AHST) in chemotherapy-responsive diseases has been tried in various neoplasms, and has resulted in higher cure rates and longer survival than conventional chemotherapy in relapsed non-Hodgkin lymphoma[3] and myeloma[4] patients. HDCT against advanced breast cancer has also shown high CR rates (up to 50%), and 10–15% of patients have enjoyed enduring remission.[5–7] However, most patients relapse following transplantation. The first reported randomized study comparing HDCT with conventional chemotherapy against advanced breast cancer showed no significant differences between the two groups in progression-free survival or overall survival.[8] Although more recent studies have shown that HDCT results in longer progression-free survival than conventional chemotherapy,[9,10] the median survival time so far appears to be no better than that achieved with conventional chemotherapy in most studies.

The reasons for the insufficient results of HDCT may be attributable to the difficulty of eradicating minimal residual disease. The patients are treated with HDCT for only 3–5 days, and further effective chemotherapy protocol does not exist because of insufficient bone marrow function after the AHST. One possible approach to overcoming the current limitation in HDCT would be multiple courses of HDCT followed by AHST, which were reported to be effective in myeloma patients.[11] But this strategy failed in a small randomized trial for metastatic breast cancer.[12] Another approach would be the transduction of autologous hematopoietic stem cells with an anticancer drug-resistance gene and reinfusion, so that normal bone marrow cells would be protected from the toxic effects of anticancer drugs. The human multidrug resistance 1 (*MDR1*) gene encodes plasma membrane P-glycoprotein (P-gp), which consists of two transmembranous domains and two ATP-binding domains. P-gp excretes various drugs such as anthracyclines, vinca alkaloids and taxanes from cells in an ATP-dependent manner.[13] The *MDR1* gene seems to be a good candidate for drug resistance gene therapy as chemotherapeutic drugs such as docetaxel and paclitaxel, which have good clinical activity in the treatment of breast cancer, are effluxed efficiently by P-gp.

Using a retroviral vector, Sorrentino *et al.*[14] transplanted *MDR1*-transduced bone marrow into irradiated mice and then treated them with paclitaxel. This treatment increased *MDR1*-transduced leukocytes in peripheral blood (*in vivo* amplification), and *MDR1*-transduced mice showed reduced bone marrow suppression by paclitaxel (bone marrow protection). Then, several groups started clinical studies of *MDR1* gene therapy against advanced breast cancer or other neoplasms.[15–17] Abonour *et al.* showed *in vivo* amplification of *MDR1*-transduced leukocytes by etoposide treatment in their clinical study on germ cell tumors.[18] Here we present the promising results of our *MDR1* gene therapy against metastatic breast cancer.

Materials and Methods

Institutional review. The whole protocol for this clinical study was reviewed and approved by our institutional review board and the governmental gene therapy committee, Japan.

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