Redox imbalance in cystine/glutamate transporter-deficient mice

Hideyo Sato§¶, Ayako Shiiya§, Mayumi Kimata§, Kanako Maebara§, Michiko Tamba§, Yuki Sakakura§†, Nobuo Makino†, Fumihiro Sugiyama#, Ken-ichi Yagami#, Takashi Moriguchi*, Satoru Takahashi*# and Shiro Bannai§

Running title: Cystine/glutamate transporter-deficient mice

§Department of Biochemistry and *Department of Anatomy and Development, Institute of Basic Medical Sciences, #Laboratory Animal Resource Center, University of Tsukuba, Tsukuba, Ibaraki 305-8575, †Center for Humanity and Sciences, Ibaraki Prefectural University of Health Sciences, Ami, Ibaraki 300-0394, ¶Department of Bioresource Engineering, Faculty of Agriculture, Yamagata University, Tsuruoka, Yamagata 997-8555, Japan

¶To whom correspondence should be addressed: Department of Bioresource Engineering, Faculty of Agriculture, Yamagata University, Tsuruoka, Yamagata 997-8555, Japan, Tel/FAX: +81-235-28-2869:
E-mail: shideyo@tds1.tr.yamagata-u.ac.jp
Cystine/glutamate transporter, designated as system x<sub>c</sub>-, mediates cystine entry in exchange for intracellular glutamate in mammalian cells. This transporter consists of two protein components, xCT and 4F2 heavy chain, and the former is predicted to mediate the transport activity. This transporter plays a pivotal role for maintaining the intracellular GSH levels and extracellular cystine/cysteine redox balance in cultured cells. To clarify the physiological roles of this transporter in vivo, we generated and characterized mice lacking xCT. The xCT<sup>+</sup> mice were healthy in appearance and fertile. However, cystine concentration in plasma was significantly higher in these mice, compared with that in the littermate xCT<sup>+/+</sup> mice, whereas there was no significant difference in plasma cysteine concentration. Plasma GSH level in xCT<sup>+</sup> mice was lower than that in the xCT<sup>+/+</sup> mice. The embryonic fibroblasts derived from xCT<sup>+</sup> mice failed to survive in the routine culture medium, and 2-mercaptoethanol was required for the survival and growth. When 2-mercaptoethanol was removed from the culture medium, cysteine and GSH in these cells drastically decreased and they started to die within 24 hours. N-acetyl cysteine also rescued xCT<sup>+/+</sup>-derived cells and permitted growth. These results demonstrate that system x<sub>c</sub>- contributes to maintaining the plasma redox balance in vivo but is dispensable in the mammalian development although it is vitally important to the cells in vitro.
Transport of amino acids across plasma membrane is mediated by several transport systems in mammalian cells (1). We have described a Na\(^+\)-independent, cystine/glutamate exchange transport system, designated as system \(x_c\), in various cultured cells like human fibroblasts and mouse peritoneal macrophages (2, 3). The cells expressing system \(x_c\) take up cystine in the medium into the cell, and reduce it to cysteine (thiol form), which is in turn used for the synthesis of GSH and proteins. A part of cysteine is released back into the medium via neutral amino acid transport systems, and the cysteine is rapidly oxidized to cystine by oxygen in the medium. Thus, a series of these transports and redox reactions constitutes cystine/cysteine cycle across the plasma membrane. The activity of system \(x_c\) contributes to driving the cystine/cysteine cycle and to maintaining the redox balance between cystine and cysteine in the culture medium (4). In the cultured cells, the activity of system \(x_c\) is also demonstrated to be essential for maintaining the intracellular GSH levels (5). Because GSH plays a central role in alleviating oxidative stress, system \(x_c\) is regarded as a constituent of the antioxidant defence systems, at least in cultured cells. This transporter is composed of two protein components, xCT and the heavy chain of 4F2 antigen (6), and the transport activity is thought to be mediated by xCT. The activity of system \(x_c\) is induced by various stimuli, including electrophilic agents like diethyl maleate (7), oxygen (4), hydrogen peroxide (8), bacterial lipopolysaccharide (LPS)\(^1\) (9), and amino acid deprivation (10). We have demonstrated that the induction of xCT by diethyl maleate is mediated by electrophile response element located in the 5' flanking region of xCT gene and that the transcription factor Nrf2 binds to this element to activate the transcription of xCT gene (11). We have recently demonstrated that the induction of xCT by amino acid deprivation is mediated by two amino acid response elements located in the 5' flanking region of xCT gene and suggested that the transcription factor ATF4 is involved in the inducible transcription of xCT gene (10).

Although it is obvious that system \(x_c\) plays a pivotal role in maintaining intracellular GSH level and modulating cystine/cysteine redox balance out of the cell \textit{in vitro}, it is unknown whether system \(x_c\) functions similarly \textit{in vivo}. To clarify the physiological role of system \(x_c\) \textit{in vivo}, we generated a mouse model deficient in xCT. In the present study, we describe the generation and initial characterization of the mice unable to express xCT.

EXPERIMENTAL PROCEDURES

\textit{Materials—}L-[\textsuperscript{14}C]Cystine was obtained from PerkinElmer Life
Sciences. Monobromobimane was purchased from Molecular Probes, Inc. (Eugene, OR). All other chemicals and agents were purchased from Sigma Chemical Co. (St. Louis, MO) or Wako Pure Chemical Industries, Ltd (Tokyo, Japan).

**Generation of xCT Null Mice**—Genomic clones containing mouse xCT gene were isolated from a 129/Sv genomic phage library (Stratagene). A 2.3-kb genomic fragment containing the translation initiation site and its 5’-flanking region was cut out with Hind III and Nco I, blunted with S1 nuclease, and inserted into modified pSVβ containing GFP coding sequence. The fragment accompanying the GFP sequence was cut out and inserted into pLOXNATA, which contains neomycin resistance (neo) and thymidine kinase for selection of homologous recombination. Another 3.9-kb genomic fragment containing parts of exon 1 and intron 1 was cut out with Nco I and Bam HI, blunted with Klenow fragment, and subcloned into pLOXNATA (Fig. 1). The targeting vector was linearized and transfected into E14 ES cells by the electroporation. The cells were cultured on growth-arrested neo embryonic fibroblasts under the G418 selection. Resistant colonies were picked on days 8-10, dissociated with trypsin, and divided into two aliquots. One aliquot was plated on a 96-well plate, and genomic DNA was isolated from another aliquot and screened by polymerase chain reaction (PCR). Through PCR analysis of approximately 1,000 ES cell clones, we identified 4 clones that carried the homologous recombinant allele. These positive clones were expanded and genotyped by Southern blot analysis. The positive ES clone was injected into C57BL/6 blastcysts and chimeric mice were generated. Male chimeric mice were bred with C57BL/6 female mice. Germ line transmission of the targeted allele was determined by the presence of agouti mice in the offspring. Mice were genotyped by isolating DNA from tail biopsies and analyzed by Southern blotting using the probes shown in Fig. 1. To remove the neo cassette in the allele of the mice, we bred these mice with Cre recombinase-expressing mice (C57BL/6). Offspring heterozygous for the neo cassette deleted allele were interbred, and mice homozygous for the mutation were identified by PCR and Southern blot analysis. All mice were 129/Svj-C57BL/6 mixed background littermates from F1 heterozygote crosses. All experiments were performed in 8-12-week-old homozygous (xCT−/−), heterozygous (xCT+/-), and wild-type (xCT+/+) littermates. This study was approved by the Animal Care and Use Committee at the University of Tsukuba.

**Cell Culture and Cystine Uptake**—Peritoneal macrophages elicited by 4% thioglycollate medium were collected and cultured as described previously (12). Embryonic fibroblasts were prepared from day 14 embryos and cultured in Iscove’s Modified Dulbecco’s Medium
supplemented with Insulin-Transferrin-Selenium-G supplement (Gibco) and 10% fetal bovine serum at 37 °C in 5% CO₂ and 95% air in the presence or absence of 50 μM 2-mercaptoethanol (2ME). For the subsequent experiments, the cells were cultured in Dulbecco’s Modified Eagle Medium supplemented with 10% fetal bovine serum with and without 2ME. Cells were plated at 2 x 10⁵ cells/35-mm diameter dish, cultured for 24 h, and the activity of cystine transport was measured as described previously (2). Briefly, cells were rinsed three times in warmed PBSG [10 mM phosphate-buffered saline (137 mM NaCl, 3 mM KCl), pH 7.4, containing 0.01% CaCl₂, 0.01% MgCl₂·6H₂O and 0.1% glucose], and then incubated in 0.5 ml of the warmed uptake medium at 37 °C for specified time periods. The uptake medium was PBSG containing L-[¹⁴C]cystine (0.05 mM and 0.1 µCi/0.5 ml). Uptake was terminated by rapidly rinsing the cells three times with ice-cold PBS, and radioactivity in the cells was determined. Cystine uptake was determined under conditions approaching initial rates of uptake, i.e. measuring uptake for cystine at 2 min. The uptake of cystine increased linearly during this incubation.

Measurement of Intracellular Cysteine and GSH—The cysteine and GSH contents in the cells were determined by the method of Cotgreave and Moldéus (13) with a slight modification (14). The cells were rapidly rinsed three times with ice-cold 20 mM HEPES-saline (137 mM NaCl, 3 mM KCl), pH 7.4, containing 0.01% CaCl₂, 0.01% MgCl₂·6H₂O and 0.1% glucose, and incubated in the dark at room temperature for 10 min with 100 μl of 8 mM monobromobimane in 50 mM N-ethylmorpholine (pH 8) and 100 μl of 50 mM HEPES-saline, containing 0.01% CaCl₂, 0.01% MgCl₂·6H₂O and 0.1% glucose. Then 10 μl of 100% trichloroacetic acid was added. The protein precipitate was removed by centrifugation at 15,000 x g for 5 min, and bimane adducts of cysteine and GSH in the supernatant were analyzed by high performance liquid chromatography (HPLC). The HPLC separation was achieved on a steel column (4.6 x 100 mm) packed with 3-μm octadecylsilica reversed-phase material. The fluorescence at 480 nm was monitored with the excitation at 394 nm. The elution was performed with 9% (vol/vol) acetonitrile in 0.25% (vol/vol) acetic acid, pH 3.7 for 8 min. The flow rate was 1 ml/min throughout the process. GSH in tissues was measured by the enzymatic method described previously (15), which is based on the catalytic action of GSH in the reduction of 5,5′-dithiobis (2-nitrobenzoic acid) by the GSH reductase system. The GSH extracted from the tissues was mostly reduced GSH, and the content of the oxidized form, GSSG, was negligible.

Northern Blot Analysis—The RNA probe for mouse xCT was digoxigenin (DIG)-labeled by transcription from the linearized plasmid using RNA-labeling mix (Roche) and T3/T7 RNA polymerase (Stratagene). RNA was
electrophoresed on a 1% agarose gel in the presence of 2.2 M formaldehyde, transferred onto positively charged nylon membrane (Roche), and hybridized with the DIG-labeled RNA probes in DIG Easy Hyb (Roche) for 16 h at 68 °C. The membranes were washed twice for 5 min at room temperature with 1 x SSC, 0.1% SDS and then washed twice for 15 min at 68 °C with 0.1 x SSC, 0.1% SDS. The hybridized bands were visualized using CDP-Star (Roche).

**Measurement of amino acid concentrations in plasma**—Mice were anesthetized with pentobarbital and blood was directly collected from the heart. Collected blood was immediately centrifuged and 100 μl of plasma was moved into the tube containing 10 μl of 50% sulfosalicylic acid and 10 nmol of norleucine as an internal standard. After 30 min in an ice bath, the mixture was frozen and stored until the assay. The frozen sample was thawed and centrifuged at 10,000 x g for 20 min. The supernatant solution was removed, its pH was adjusted to 2.0 with 1 M LiOH, and 50 μl of the solution was assayed by the amino acid analyzer (JLC-300, JEOL Ltd, Japan). In this analysis, cysteine was eluted coincidently with α-aminoadipate and could not be determined.

**Measurement of cysteine, GSH, and the non-protein-bound disulfide forms of cysteine and those of GSH in plasma**—To determine cysteine and GSH concentrations, 100 μl of the plasma was immediately mixed with 100 μl of 8 mM monobromobimane in 50 mM N-ethylmorpholine, pH 8, and incubated in the dark at room temperature for 10 min. Then 10 μl of 100% trichloroacetic acid was added. The protein precipitate was removed by centrifugation at 15,000 x g for 5 min, and 20μl of the supernatant was measured by HPLC as described above. Concentrations of the total non-protein-bound disulfide forms of cysteine (CySST) and those of GSH (GSST) were determined as described previously (16). Briefly, plasma was treated with N-ethyl maleimide to block free thiol compounds and then deproteinized by adding sulfosalicylic acid. Precipitated protein was removed by centrifugation and NaBH₄ was added to the supernatant to reduce disulfide compounds. The excess N-ethyl maleimide is inactivated by NaBH₄. The thiols formed were derivatized with monobromobimane and the bimane adducts of cysteine and GSH were analyzed by HPLC. CySST is equal to cystine + CySSX, where CySSX is the mixed disulfide of cysteine and the other thiols, and GSST is equal to GSSG + GSSY, where GSSY is the mixed disulfide of GSH and the other thiols. Cysteinylglycine and homocysteine may be included as a moiety in the mixed disulfides. However, the bimane adducts of cysteinylglycine and homocysteine were very small in quantity in HPLC analysis, and therefore, most of CySSX and GSSY was presumed to be CySSG.

**RESULTS**
Generation of xCT Null Mice—Mice heterozygous for targeted disruption of the xCT gene were obtained by crossing the chimeric males with wild-type female mice and agouti offspring were screened for the presence of mutated xCT gene by PCR. Offspring heterozygous for the mutation were interbred, and mice homozygous for the xCT disrupted allele were identified by Southern blot analysis (Fig. 2A). In the macrophages derived from these mice, however, aberrant mRNA for xCT was transcribed and the significant activity of system x⁻ was detected, although the induction of the activity of system x⁻ by LPS was significantly decreased (Fig. 2B and C). Primer extension and 5’ rapid amplification of cDNA ends experiments demonstrated that the aberrant transcripts seemed to be initiated in the neoţ cassette which was inserted in the opposite orientation between the xCT genomic sequences (data not shown).

We bred these mice with Cre recombinase-expressing mice to remove the neoţ cassette (Fig. 1D). Offspring heterozygous for the neoţ cassette deleted allele were interbred, and mice homozygous for the mutation were identified by PCR and Southern blot analysis (Fig. 3A). In these homozygous mice (-/- in Fig. 3), the expression of mRNA for xCT was completely abolished in brain and thymus where xCT mRNA is constitutively expressed in the wild-type mice (Fig. 3B). In the peritoneal macrophages derived from these mice, no xCT mRNA was detected (Fig. 3C). Activity of cystine transport was slightly detected, but the activity was not inhibited by glutamate and was not induced by LPS, indicating that no activity of cystine transport via system x⁻ is expressed in these cells (Fig. 3D). These data confirmed that these homozygous mice are xCT null mutants.

Analysis of xCT Null Mice—These xCT⁻ mice developed normally and both males and females were fertile. They were healthy in appearance at the age of 6 months. The average litter size of homozygous B6;129 mixed background mutants were 8.2 ± 2.3 (mean ± S.D., n = 10). The percentage of the 8-week-old xCT⁻ progeny from xCT+⁻ parents with mixed B6;129 background was 23% (n = 81), indicating that neither peri⁻ nor postnatal death rates in the mutants were abnormal. Microscopically no abnormalities were found in any of the organs, including brain, lung, liver, heart, spleen, pancreas, thymus, intestine, adrenal glands, thyroid glands, skeletal muscle, esophagus, stomach, kidney, urinary bladder, uterus, ovary, and testis of mutants and their controls at the age of 8 weeks (data not shown). In haematological investigations, no abnormalities were found in these mice (data not shown).

Analysis of plasma amino acids from littermate xCT+/-, xCT+⁻, and xCT⁻ mice revealed that the xCT⁻ homozygotes contained approximately double the concentration of cystine in their plasma relative to the wild type
mice (Fig. 4). Plasma amino acid concentrations other than cystine showed no significant differences. Cysteine concentration was similar in the plasma of xCT+/+, xCT+/-, and xCT−/− mice, whereas the xCT−/− homozygotes contained approximately half the concentration of GSH of the wild type mice (Fig. 5). Concentration of the total non-protein-bound disulfide forms of cysteine (CySST, i.e., cystine + CySSX) in plasma was much higher in xCT−/− mice than in xCT+/− mice. Considering the cystine concentration shown in Fig. 4, the major part of CySST is thought to be cystine. GSST (GSSG + GSSY) was slightly higher in xCT−/− mice but the sum of GSST and GSH was nearly equal in xCT+/+, xCT+/-, and xCT−/− mice. The results suggest that the plasma of xCT−/− mice is in more oxidized state than that of wild type mice. We have measured the GSH contents in the liver, kidney, cerebrum, cerebellum, thymus, and spleen of xCT+/+ and xCT−/− mice at the age of 8-12 weeks old, and could not find any significant differences between the two groups (data not shown).

Characterization of Embryonic Fibroblasts Derived from xCT Null Mice—We tried to culture embryonic fibroblasts from the day 14 embryos of the littermate xCT+/+, xCT+/-, and xCT−/− mice. The cells derived from xCT−/− mice did not proliferate and mostly died under the routine culture conditions, whereas the cells derived from xCT+/+ and xCT+/- mice well proliferated. However, when xCT−/− cells were cultured in the presence of with 50 μM 2ME, the cells proliferated normally (Fig. 6). In these embryonic fibroblasts, the activity of cystine transport of the xCT−/− cells was very low, compared with that of the xCT+/+ and xCT+/- cells (Fig 7A), and the activity showed Na+-dependency and was not inhibited by glutamate (data not shown), indicating that the slight transport of cystine in the xCT−/− cells was not mediated by system x c-. In these cells, the intracellular cysteine and GSH of xCT−/− cells were drastically decreased within 8 h in the absence of 2ME (Figs. 7B and C).

Cell proliferation was examined in the presence of N-acetyl cysteine (NAC) or vitamin E. As shown in Fig. 8, NAC caused the proliferation of the xCT−/− cells cultured in the routine culture condition without 2ME. However, the effective concentration of NAC for maintaining the cell growth was more than 1 mM, which was much higher than that of 2ME. On the other hand, vitamin E protected xCT−/− cells from the death induced by the withdrawal of 2ME, although the cells did not proliferate.

DISCUSSION

In the present study, we describe the generation of a null mutation of xCT gene by homologous recombination in the mouse. The mutation results in the redox imbalance in plasma of the mice, i.e., a significant oxidative shift of the
plasma cystine/cysteine redox balance and the decrease in the plasma concentration of GSH, compared with those of the wild type mice. As Jones, et al. have described (17), the redox potential value ($E_h$) for the cystine/cysteine couple (cystine + 2H$^+$ + 2e$^-$ $\leftrightarrow$ 2cysteine) is calculated using Nernst equation,

$$E_h = E_0 + \frac{RT}{nF} \ln \left( \frac{[\text{cystine}]}{[\text{cysteine}]^2} \right)$$

where $E_0$ (in mV) is the standard potential for the redox couple, $R$ is the gas constant, $T$ is the absolute temperature, $n$ is the number of electrons transferred, and $F$ is Faraday’s constant. $E_0$ value for the cystine/cysteine couple (pH 7.4) is –250 mV (18). $E_h$ values are equivalent to REDST values ([acid soluble thiol]$^2$/[cystine]) defined by Hildebrandt, et al (19). As shown in Figs. 4 and 5, the plasma concentrations of free cystine and cysteine are 33 µM and 18.4 µM on average, respectively, in the xCT$^{+/+}$ mice. The $E_h$ value in these mice is approximately –100 mV. On the other hand, in the xCT$^{-/-}$ mice, the concentrations of free cystine and cysteine are 82 µM and 18.6 µM on average, respectively, and $E_h$ value is approximately –89 mV. The oxidation change between the xCT$^{+/+}$ and xCT$^{-/-}$ mice is 11 mV. Jones, et al., showed that the $E_h$ value for cystine/cysteine redox balance in the human subjects at the age of around 20 years old is approximately –80 mV and a linear oxidation of cystine/cysteine redox balance occurs with age at a rate of 0.16 mV/year over the entire age span (17). The oxidative shift of $E_h$ value in the xCT$^{-/-}$ mice may imply that the ageing is accelerated in these mice. Of particularly interest is the prominent increase in plasma cystine in elderly subjects with a little change in plasma cysteine (17). It is noteworthy that the similar results are observed between the xCT$^{+/+}$ and xCT$^{-/-}$ mice at the ages of 8-10 weeks old, i.e., significantly higher plasma cystine with remaining unchanged in the plasma cysteine in the xCT$^{-/-}$ mice, compared with the xCT$^{+/+}$ mice.

It is thought that the plasma concentrations of cystine and cysteine are largely determined by several mechanisms, including the interaction of cysteine from dietary proteins or endogenous protein catabolism with oxidized form of albumin and with the intracellular GSSG pool of erythrocytes, and the membrane transport activities and clearance rates of cystine and cysteine (20). Especially, the activity of the reabsorption system of cystine expressed in the proximal tubule of the kidney is an important factor for regulating the plasma cystine and cysteine concentrations (21). In the plasma of patients with end stage renal failure, significant elevation of cystine is observed before dialysis (22, 23). Thus, the reabsorption system of cystine expressed in the kidney, which is mediated by amino acid transport system $b^{0,+}$, probably plays a pivotal role for maintaining the plasma cystine/cysteine redox balance. However, the results presented here suggest that xCT also functions as a
system for the clearance of cystine in plasma. xCT mRNA is constitutively expressed in some specific regions of the brain (24), in thymus (Fig. 3B) and in spleen (unpublished data). These organs may take part in the clearance of cystine in plasma. Because xCT⁻/⁻ mice develop normally, system b₀⁺ may compensate at least in part the function of system x₀⁻ in xCT⁺/⁺ mice.

In GSH contents in tissues so far determined, there was no difference between xCT⁺/+ and xCT⁻/⁻ mice. Presumably, it is due to unchanged plasma cysteine concentration. On the other hand, the plasma GSH level is significantly decreased in xCT⁻/⁻ mice. It may be that cystine at the relatively high concentration in plasma of these mice reacts with GSH to form cysteine/GSH mixed disulfide (CySSG), resulting in the decreased GSH. This view is supported by the data shown in Fig. 5 where the decrease of GSH in xCT⁻/⁻ mice was compensated by the increase of GSST. No significant difference in the sum of the plasma concentrations of GSH and GSST may exclude a possibility that xCT⁻/⁻ mice have a defect in hepatic GSH export.

The fibroblasts from the embryo of the xCT⁻/⁻ mice could not survive under the routine culture conditions, but they grew normally in the presence of 2ME (Fig. 6). As described previously (25), 2ME reacts with cystine in the medium and produces a mixed disulfide of 2ME and cysteine, which is taken up by the cells through neutral amino acid transporters. The mixed disulfide in the cells is rapidly reduced to cysteine and 2ME, and the latter is released out of the cells and reacts with cystine again. In this way, cysteine is supplied and the cells can survive and grow. Because 2ME functions catalytically, 50 µM 2ME is enough to provide cysteine. On the other hand, relatively high concentration of NAC is required for maintaining the proliferation of the cells derived from xCT⁺/⁺ mice (Fig. 8). NAC is thought to be transported into the cell directly and is converted to cysteine. Compared with the cells cultured with 2ME or NAC, the cells cultured with vitamin E did not grow though they survive (Fig. 8). GSH in these cells was almost undetectable (data not shown). In this case, cysteine is not supplied from the medium and the cells cease to grow because of the inhibition of the net protein synthesis. The results suggest that the cells from xCT⁻/⁻ mice under the routine culture conditions die due to oxidant stress, which is alleviated by an antioxidant such as GSH or vitamin E.

xCT is a highly inducible protein, i.e., it is induced by electrophilic agents (7), oxygen (4), and some stimuli like LPS and TNF-α (9). The xCT⁻/⁻ mice may be more sensitive to oxidative stress. It is worth investigating the response of these mice to these stresses in vivo. The oxidative shift of the plasma cystine/cysteine redox balance was observed not only in association with increased age (17, 26) but also in patients in cancer and in the people who are infected with HIV or smoke (26-28). Various signal cascades have been found in response to moderate...
changes in the redox state (29). The plasma cystine/cysteine redox balance is significantly oxidized in the xCT−/− mice even at the age of 8-10 weeks old. It is likely that some signal cascades are modulated in these mice. The xCT−/− mice may be useful to investigate the senescence and the diseases related to oxidative stress.

Acknowledgment—We are grateful to Drs. M. Masu, K. Keino-Masu, S. Hisano (University of Tsukuba) for their thoughtful discussions and Drs. G. W. Bornkamm and M. Conrad (GSF, Munich) for their helpful suggestions. We thank Dr. K. Araki (Kumamoto University) for providing the Cre recombinase-expressing mice (Ayu-1-Cre).

REFERENCES

Footnotes

This work was supported by a grant-in-aid for scientific research from the Ministry of Education, Science, Sports and Culture in Japan.

1The abbreviations used are: LPS, lipopolysaccharide; PCR, polymerase chain reaction; PBSG, phosphate-buffered saline containing 0.01% CaCl₂, 0.01% MgCl₂·6H₂O and 0.1% glucose; HPLC, high performance liquid chromatography; HEPES, N-(2-Hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid); CySST, Total non-protein-bound disulfide forms of cysteine; CySSX, the mixed disulfide of cysteine and the other thiols; GSST, Total non-protein-bound disulfide forms of GSH; GSSY, the mixed disulfide of GSH and the other thiols; 2ME, 2-mercaptoethanol; NAC, N-acetyl cysteine.
Figure Legends

Fig. 1 Gene targeting strategy. Targeting scheme for xCT disruption. The genomic structure of mouse xCT gene is presented at the top (A). The targeting vector (B) was designed such that the GFP + Neo cassette replaced the Nco I (N) fragment in exon 1. The predicted mutant allele is shown in (C). The portions of 5’ and 3’ probes used for Southern blot analysis are shown by bars. After crossing the homozygous recombinant mice with Cre-recombinase expressing mice, the predicted allele in which the Neo cassette was deleted is shown in (D). RI, Eco RI site; Ac, Acc I site; Sp, Spe I site; H, Hind III site; N, Nco I site; B, Bam HI site.

Fig. 2 Characterization of the homozygous recombinant mice. A, Southern blot analysis of genomic DNA isolated from mouse tails derived from the homozygous recombinant mice (Homo) which possess the mutant allele shown in Fig. 1C or from the wild type mice (Wild) using 5’ and 3’ probes. DNA was digested with Eco RI. The restriction enzyme sites of Eco RI occur in the Neo cassette. B, Northern blot analysis of total RNA of the macrophages derived from the mice which possess the mutant allele shown in Fig. 1C (Homo) or the wild type mice (Wild). The cells were cultured for 1 or 8 h with or without 1 ng/ml LPS, then RNA was isolated. Northern blot analysis was performed using the DIG-labeled RNA probe for mouse xCT cDNA. C, The rate of uptake of [14C]cystine in the macrophages derived from the mice which possess the mutant allele shown in Fig. 1C (Homo) or the wild type mice (Wild). The cells were cultured for 1 or 12 h with or without 1 ng/ml LPS, then the rate of uptake of cystine was measured in the presence (solid bar) or absence (open bar) of 2.5 mM glutamate. Data represent the means ± S.D. (n=4-6).

Fig. 3 Characterization of xCT−/− mice. A, Southern blot analysis of genomic DNA isolated from mouse tails derived from the homozygous recombinant mice which were crossed with Cre-recombinase expressing mice and possess the mutant allele shown in Fig. 1D using 5’ and 3’ probes (+/−). DNA was digested with Acc I (for 5’ probe) and Spe I (for 3’ probe). The restriction enzyme site of Acc I occurs in the GFP region. B, Northern blot analysis of total RNA of brain and thymus derived from the mice which possess the mutant allele shown in Fig. 1D (+/−) or the wild type mice (+/+) . Northern blot analysis was performed using the DIG-labeled RNA probe for mouse xCT cDNA. C, Northern blot analysis of total RNA of the macrophages derived from the mice which possess the mutant allele shown in Fig. 1D (+/−) or the wild type mice (+/+). The cells were cultured for 1 or 8 h with or without 1 ng/ml LPS, then RNA was isolated. Northern blot analysis was performed using the DIG-labeled RNA probe for mouse xCT cDNA. D, The rate of uptake of cystine in the macrophages derived from the mice which possess the mutant allele shown in Fig. 1D (+/−) or the wild type mice (+/+). The cells were
cultured for 1 or 12 h with or without 1 ng/ml LPS, then the rate of uptake of [14C]cystine was measured in the presence (solid bar) or absence (open bar) of 2.5 mM glutamate. Data represent the means ± S.D. (n=4-6).

**Fig. 4** Concentrations of amino acids in the plasma of the xCT⁺/⁺, xCT⁺/−, and xCT⁻/⁻ mice. Plasma of littermate xCT⁺/⁺ (open bar), xCT⁺/− (hatched bar), and xCT⁻/⁻ (solid bar) mice at the age of 8-10 weeks old were isolated, and the concentrations of amino acids were measured by the amino acid analyzer. The magnification of the data on the concentrations of valine, cystine (CYSS), and methionine was shown in the inset. Data represent the means ± S.D. (n=4). TAU, taurine; CIT, citrulline; CYST, cystathionine; ORNI, ornithine; HPRO, hydroxyproline.

**Fig. 5** Concentrations of cysteine, GSH, CySST, and GSST in the plasma of the xCT⁺/⁺, xCT⁺/−, and xCT⁻/⁻ mice. Plasma of littermate xCT⁺/⁺ (open bar), xCT⁺/− (hatched bar), and xCT⁻/⁻ (solid bar) mice at the age of 8-10 weeks old were isolated, and concentrations of cysteine, GSH, CySST (cystine + CySSX), and GSST (GSSG + GSSY) were measured. Concentrations of CySST and GSST are expressed as cysteine equivalent and GSH equivalent, respectively. Data represent the means ± S.D. (n=4-10). *p < 0.05 (relative to xCT⁺/⁺ mice), **p < 0.01 (relative to xCT⁺/⁺ mice).

**Fig. 6** Proliferation of the embryonic fibroblasts derived from the xCT⁺/⁺, xCT⁺/−, and xCT⁻/⁻ mice. The cells derived from xCT⁻/⁻ mice were seeded in 35-mm diameter dish and cultured in the presence of 50 µM 2-ME for 1 day. Then, the medium was replaced with fresh one with (●) or without (○) 50 µM 2-ME (Day 0). Nigrosin-excluding cells were counted at 24-h intervals. Data represent the means ± S.D. (n=4).

**Fig. 7** The activity of cystine uptake (A), and intracellular cysteine (B) and GSH (C) in embryonic fibroblasts derived from the xCT⁺/⁺, xCT⁺/−, and xCT⁻/⁻ mice. A, xCT⁺/⁺ and xCT⁺/− cells were cultured in the absence of 50 µM 2ME, and xCT⁻/⁻ cells were cultured in the presence of 50 µM 2ME. Then, rate of uptake of [14C]cystine was measured. Data represent the means ± S.D. (n=4). B and C, xCT⁺/⁺ and xCT⁺/− cells were cultured in the absence of 50 µM 2ME, and xCT⁻/⁻ cells were cultured in the presence of 50 µM 2ME. Then, the cells were cultured in the medium with (●) or without (○) 50 µM 2ME, and the cell extract was prepared at the time point indicated. The concentrations of intracellular cysteine and GSH were measured. Data represent the means ± S.D. (n=4).
Fig. 8 Effect of NAC and vitamin E on the cell proliferation. The embryonic fibroblasts derived from the xCT<sup>+</sup>++, xCT<sup>+</sup>−, and xCT<sup>−</sup> mice were seeded at 1 x 10<sup>5</sup> cell/35-mm diameter dish and cultured with no additives (solid bar), 50 µM 2ME (open bar), 1 mM NAC (hatched bar), or 1 µg/ml vitamin E (grey bar) for 48 h. Then, nigrosin-excluding cells were counted. Data represent the means ± S.D. (n=4).
**Fig. 1**

Diagram showing genomic elements and probes:

- **A** represents the genomic region with Exon1 and Exon2, separated by loxP sites.
- **B** illustrates the recombination event with MC1+Neo+MC1+TK cassette flanked by loxP sites.
- **C** and **D** show the 5' and 3' probe regions, respectively, adjacent to the recombinant cassette.
Fig. 2
Fig. 3

A

B

C

D

Rate of uptake of cystine (nmol/min/mg protein)

+/-  -/-  +/-  +/+ (Kb)

0 0.5 1 1.5 2 2.5

1 h 12 h LPS 1 h 12 h LPS

+/-  -/-
Amino acid concentration (mM)

Fig. 4
Fig. 5
Cell number (×10⁶ cells/dish) vs. Time (day)
Fig. 7

(A) Rate of uptake of cystine (nmol/min/mg protein)

(B) Concentration of cysteine (nmol/mg protein)

(C) Concentration of GSH (nmol/mg protein)
Redox imbalance in cystine/glutamate transporter-deficient mice
Hideyo Sato, Ayako Shiiya, Mayumi Kimata, Kanako Maebara, Michiko Tamba, Yuki Sakakura, Nobuo Makino, Fumihiro Sugiyama, Ken-ichi Yagami, Takashi Moriguchi, Satoru Takahashi and Shiro Bannai

J. Biol. Chem. published online September 6, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M506439200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts