xCT deficiency accelerates chemically induced tumorigenesis

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During the course of inflammation and its resolution, macrophages are exposed to various cytotoxic materials, including reactive oxygen species. Thus, macrophages require a protective machinery against oxidative stress to survive at the inflammatory site. Here, we showed that xCT, a component of transport system \(x^{-\text{c}}\), was significantly up-regulated in activated infiltrating cells, including macrophages and neutrophils at the inflammatory site. System \(x^{-\text{c}}\) mediates the uptake of extracellular L-cystine and is consequently responsible for maintenance of intracellular glutathione levels. We established a loss-of-function mouse mutant line of xCT by N-ethyl-N-nitrosourea mutagenesis. Macrophages from xCT\textsuperscript{mu/mu} mice showed cell death in association with the excessive release of high mobility group box chromosomal protein 1 upon stimulation with LPS, suggesting that xCT deficiency causes unremitting inflammation because of the impaired survival of activated macrophages at the inflammatory site. Subcutaneous injection of 3-methylcholanthrene (3-MCA) induced the generation of fibrosarcoma in association with inflammation. When 3-MCA was injected s.c. into mice, xCT mRNA was up-regulated in situ. In xCT\textsuperscript{mu/mu} mice, inflammatory cytokines (such as IL-1\(\beta\) and TNF\(\alpha\)) were overexpressed, and the generation of 3-MCA-induced fibrosarcoma was accelerated. These results clearly indicate that the defect of the protective system against oxidative stress impaired survival of activated macrophages and subsequently enhanced tumorigenecity.

Several lines of evidence have demonstrated that chronic inflammation initiates and promotes cellular transformation, tumorigenesis, and tumor progression (1–4). In humans, cancer is frequently associated with chronic inflammation induced by persistent bacterial and viral infection in several organs. Inflammatory bowel disease, such as ulcerative colitis, is associated with an elevated risk of colorectal cancer. Such a relationship between chronic inflammation and carcinogenesis is also confirmed in mouse (5, 6). The molecular mechanisms of inflammation-induced tumorigenesis have been extensively studied in mice, and several inflammatory-related molecules have been found to be necessary for carcinogen-induced tumorigenesis in mouse (7–10).

Macrophages are key players in the initiation and regulation of inflammation. At the inflammatory site, macrophages infiltrating in an acute injury or infected sites recognize invading microorganisms or endogenous adjuvants released by injured cells, such as high mobility group box chromosomal protein 1 (HMGB1) and uric acid, through pattern-recognition receptors (11). In response to this recognition, macrophages produce inflammatory cytokines and chemokines. Although the precise mechanisms of inflammation-induced tumorigenesis remain unclear, cytokines or growth factors persistently produced at the chronic inflammatory site may stimulate the proliferation of DNA-damaged cells.

Macrophages are also required for the resolution of inflammation, which is critical for successful tissue repair (12). During the late course of inflammation, infiltrated neutrophils and cell debris from tissue injury are cleared by macrophages. A defect of this clearance has been shown to cause unremitting inflammation in acute-injury mouse models (13–15). Therefore, the impairment of macrophage function in the late course of inflammation may cause chronic persistent inflammation and subsequent tumorigenesis.

During the course of inflammation and its resolution, macrophages are exposed to various cytotoxic materials, including reactive oxygen species (ROS). In the active phase of inflammation, neutrophils are recruited to the inflammation site, where they produce ROS to kill the invading organisms. Macrophages themselves also produce ROS in response to LPS (16, 17). From this point of view, macrophages require a protective machinery against oxidative stress to survive and accomplish the task of resolving inflammation. However, the molecular mechanisms and physiological roles of the antioxidant system in macrophages remain unclear.

In the present study, we demonstrated that xCT, a component of transport system \(x^{-\text{c}}\) that mediates the uptake of extracellular L-cystine coupled to L-glutamate efflux, was expressed in activated infiltrating cells, including macrophages and neutrophils, at the inflammatory site. As the uptake of cystine and its subsequent reduction to cysteine are essential for the synthesis of glutathione (GSH), system \(x^{-\text{c}}\) is responsible for maintenance of intracellular GSH levels. GSH plays a prominent role in cellular defense against ROS produced under various conditions, including inflammation. Consistent with this, xCT deficiency caused abnormal death of activated macrophages in association with release of an excess amount of HMGB1. Unremitting inflammation accelerated chemically induced tumorigenesis in xCT-mutant mice. These findings demonstrate that the antioxidant system in activated macrophages is required for the prevention of unremitting inflammation, which contributes to cellular transformation and tumorigenesis.

\textbf{Results}

\textit{xCT Mutant Mice Exhibit Failure in In Vitro Macrophage Generation.}

Third generation (G3) C57BL/6 mice homozygous for random mutation induced by N-ethyl-N-nitrosourea (ENU) were screened for the generation and function of macrophages and dendritic cells (DCs). E17.5 G3 embryonic liver cells containing hematopoietic progenitor cells were stimulated with GM-CSF to generate DCs. In the G3 mice derived from a pedigree (no. 8), 7 of 64 embryos showed no proliferation of DCs in response to GM-CSF. The G3 mice from the same pedigree were further sib-mated or

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mated with WT C57BL/6 mice to establish the mutant mouse line. As shown in Fig. 1, two out of nine newborn mice from the mating of the possible heterozygous male and female mice showed no proliferation of either DCs or macrophages stimulated with GM-CSF or M-CSF, respectively. We also checked bone marrow cells from mutant adult mice for the generation of DCs and macrophages. As found in embryo or newborn liver cells, bone-marrow cells from the mutant did not respond to the cytokines in terms of generation of DCs and macrophages.

The mutation in the line was mapped in the C3H/HeJ mouse strain. F2 mice were phenotyped by generating of DCs from newborn livers. Analysis of n=200 F2 mice revealed that the mutation was confined to a position between 49.9 and 71.5 Mb on chromosome 3. In this position, a gene encoding xCT or SLC7A11, a subunit of cysteine/glutamate transporter (18), is located. The xCT gene consists of 12 exons and the sequence of each exon was examined. Sequencing of xCT in the mutant mice revealed a G-to-T transversion at the thirty-first base in exon 10 (Fig. 2A). This transversion caused premature termination at position 383 glycine (Fig. 2B). xCT is predicted to have a 12-transmembrane domain (19), and the mutation is located in the ninth transmembrane region. As analysis of the subtel gray (sut) mice revealed that the C-terminal region of xCT encoded by exon 12 was essential for its function (20), it was most likely that xCT was the loss-of-function mutation. We confirmed that all of the mice carrying the homozygous mutation exhibited the DC- and macrophagenonproducing phenotype with a frequency of n=25%, in accordance with the recessive pattern of inheritance and the Mendelian Law.

The transport system xCT is responsible for the maintenance of intracellular GSH levels and is up-regulated on exposure to ROS (21). Embryonic fibroblasts derived from xCT-deficient mice required 2-mercaptoethanol (2-ME) in culture for survival (22), and the same phenotype was observed in embryonic fibroblasts from the xCT mutant mice (Fig. S1). Thus, we examined whether 2-ME supported the growth of bone marrow-derived cells from the mutant mice. Bone marrow-derived cells could be generated from xCT mutant bone marrow cells in response to GM-CSF only in the presence of 2-ME (Fig. 2C, filled bar). These results indicate that xCT plays a critical role in the in vitro generation of macrophages and DCs from hematopoietic progenitor cells by maintaining intracellular levels of GSH.

Fig. 1. There is no generation of dendritic cells and macrophages from hematopoietic progenitor cells of liver of no. 8 pedigree of ENU mutant newborns. G4 newborns were obtained by mating of the male and female xCT mice from the no. 8 pedigree. Liver cells obtained from G4 newborns were cultured with either GM-CSF or M-CSF. Note that liver cells of two of nine newborn mice (both were homozygous mutants of xCT) showed no proliferation of either DCs or macrophages. Genotype of xCT in each mouse was determined, and is shown in the pedigree tree (WT, +/+; heterozygous, +/m; homozygous, m/m).

xCT Is Not Required for in Vivo Homeostasis of Macrophages and DCs in Steady-State Conditions. We next examined the in vivo generation of these cells in xCT mutant mice. Splenocytes from heterozygous and homozygous mutants were analyzed by flow cytometry. As shown in Fig. 3A, populations of CD11b+ macrophages and CD11c+ DCs were not different between homozygous and heterozygous xCT mutant mice. We further examined the populations of...
DC subsets in spleens of xCT mutant mice. CD11c+ cells were enriched with magnetic beads, and analyzed by flow cytometry. As shown in Fig. 3B, we could not see any abnormalities in the populations of CD8α+ and CD8α− conventional DCs and B220+ plasmacytoid DCs.

We also analyzed macrophage population in the peritoneal cavity, and found that there were no differences in the number of peritoneal resident macrophages between xCTmut+/+ and xCTmut/mut mice (Fig. 3C). In addition, we did not observe any abnormality either in myeloid cell population of peripheral blood or those of bone marrow in xCTmut/mut mice (Fig. S2). These results indicate that xCT is dispensable for the in vivo homeostasis of macrophages and DCs under steady-state conditions.

**xCT mRNA Expression Is Enhanced in Infiltrating Cells at the Inflammatory Site.** As described above, although xCT is definitively required for the generation of macrophages and DCs from bone marrow progenitor cells in vitro, there was no abnormality in the in vivo population of these cells in xCT homozygous mutant mice. Consistent with these findings, xCT mRNA was not detectable in thioglycollate-elicited peritoneal macrophages (Fig. 4A). On the other hand, LPS stimulation dramatically increased the levels of xCT mRNA. These results suggest that xCT may play a critical role in inflammation-related conditions. To further explore this possibility, we analyzed the expression of xCT mRNA in some inflammatory conditions. We first employed an experimental model of hepatitis induced by combination of *Propionibacterium acnes* and LPS. In this model, LPS injection rapidly induced hepatocellular injury in *P. acnes*-sensitized mice. As shown in Fig. 4B, xCT mRNA levels were dramatically increased 2 h after injection of LPS. We further tested the expression of xCT mRNA in bleomycin-induced pneumonia, another experimental model of inflammatory diseases. In this model, the intratracheal injection of bleomycin induced acute inflammatory pneumonia. xCT mRNA levels were dramatically increased 30 h after bleomycin injection (Fig. 4C). We further examined cells that mainly expressed xCT mRNA in the bleomycin-induced pneumonia model. Five days after injection of bleomycin, infiltrating leukocytes, possibly including neutrophils and macrophages, were observed mostly in the perivascular region. In situ hybridization analysis revealed that xCT mRNA was abundantly expressed in these infiltrating cells (Fig. 4D). On the other hand, hybridization signals were hardly detected in parenchymal cells, including alveolar epithelial cells and bronchial epithelial cells. Taken together, these results clearly indicate that xCT is dominantly expressed in the infiltrating immune cells at the acute inflammation site.

**Activated Macrophages from xCTmut/mut Mice Show Impaired Survival.** To explore the role of xCT in macrophages in inflammatory conditions, we next examined the consequence of xCT deficiency in LPS-stimulated peritoneal macrophages. Thioglycollate-elicited peritoneal macrophages were prepared from xCTmut+/+ and xCTmut/mut mice. Upon stimulation with LPS, macrophages from xCTmut/mut mice produced TNFα at the same levels as those from xCTmut+/+ mice (Fig. 5A), indicating that xCT is not required in the cytokine expression. However, 72 h after LPS stimulation, morphological changes were observed in the macrophages from xCTmut/mut mice, most of the xCTmut/mut macrophages became rounded with an irregular surface and were detached from the culture dish. Some cells were ruptured (Fig. 5B). Consistent with these morphological changes, the viability of macrophages from xCTmut/mut mice was greatly reduced compared with that of WT macrophages (Fig. 5C). These results indicate that xCT is required for the survival of activated macrophages. LPS stimulation inducing oxidative stress in the macrophages has been reported (17). Thus, it is most likely that xCT plays a critical role in protecting macrophages from the oxidative stress induced by LPS.

We further examined the type of cell death occurring in LPS-stimulated macrophages from xCT mutant mice. To examine whether the macrophages died via apoptosis, we first examined caspase activation in LPS-stimulated macrophages from xCT mutant mice using a fluorescence-labeled active caspase-binding substrate, but no activation of caspasess was observed in LPS-stimulated macrophages from xCT mutant mice (Fig S3). We next examined chromosomal DNA fragmentation, one of the most common features of apoptosis, in these cells. Consistent with the absence of caspase activation, such a DNA ladder was not observed in LPS-stimulated macrophages from xCT mutant mice (Fig. S4). These results clearly indicate that the apoptotic cell death pathway was not activated in LPS-stimulated macrophages from xCT mutant mice. To further clarify the mode of cell death in these macrophages, we performed electron microscopic observation. Forty-eight hours after LPS stimulation, most macrophages from xCT mutant mice seemed to undergo necrotic cell death, demonstrated by the degradation of organelles including nucleus, loss of plasma membrane integrity, and leakage of cellular contents (Figs. S5 B and D). Some cells showed accelerated autophagy (Fig. S5C). Chromatin condensation and fragmentation in nuclei, the most common morphological feature of apoptotic cells, were rarely observed. Taking these results into consideration, we can deduce that LPS-stimulated macrophages from xCT mutant mice did not undergo apoptosis but possibly necrotic cell death. HMGB1 is passively released from injured cells, and exhibits a variety of inflammation-promoting activities (23). We next measured the concentration of HMGB1 in the culture supernatant of LPS-stimulated macrophages. As HMGB1 is actively secreted by macrophages upon stimulation with LPS (24), HMGB1 was detectable in WT macrophages (Fig. 5D). In contrast, a large amount of HMGB1 was detected in the supernatants of macrophages from xCTmut/mut mice. It is most likely that excess amount of HMGB1 in LPS-stimulated macrophages from xCT deficient mice was the result of passive release from necrotic macrophages. These
Enhanced expression of xCT mRNA in activated macrophages. (A) xCT expression in LPS-stimulated macrophages. Thiglycollate-elicited peritoneal macrophages from C57BL/6 mice were stimulated with 0.1 μg/mL LPS for the indicated time. After stimulation, total RNA was prepared from these cells and the amount of xCT mRNA was measured by quantitative RT-PCR. Relative amount of xCT mRNA are shown with SD. *, P < 0.01. (B and C) xCT expression in an experimental model of hepatitis induced by a combination of P. acnes and LPS. Five hundred micrograms of P. acnes was injected i.v. into C57BL/6 mice. Eight days later, the mice were injected i.p. with 20 μg of LPS for induction of hepatitis. Two hours after LPS injection, total RNA was prepared from livers, and the amounts of xCT mRNA was measured by quantitative RT-PCR. Relative amount of xCT mRNA are shown with SD. *, P < 0.01. (C and D) xCT expression in bleomycin-induced pneumonitis. Mice were injected intratracheally with 0.1 U bleomycin. (C) Thirty hours later, total RNA was prepared from lungs and the amount of xCT mRNA was measured by quantitative RT-PCR. Relative amount of xCT mRNA are shown with SD. *, P < 0.01. (D) Five days after bleomycin injection, localization of xCT mRNA in lungs was analyzed by in situ hybridization. The serial sections of control lung (i, iii, and v) or bleomycin-treated lung (ii, iv, vi, and vii) were stained with H&E (i and ii), in situ hybridization with a sense probe (iii and iv), or an antisense probe (v–vii) for xCT mRNA. Panel vii is a magnification of the boxed region in vi. Original magnification: 100× (i–vi), 400× (vii). (Scale bar, 50 μm.)

**Fig. 5.** Impaired survival of activated macrophages from xCT+/− mice. (A) Thioglycollate-elicited peritoneal macrophages from xCT+/+ or xCT−/− mice were stimulated with 0.1 μg/mL or 1 μg/mL of LPS for 24 h. Production of TNFα was measured by ELISA. Mean values are shown with SD. (B and C) Thioglycollate-elicited peritoneal macrophages from xCT+/+ or xCT−/− mice were stimulated with 1 μg/mL of LPS. Seventy-two hours after stimulation, these macrophages were observed under a microscope (B). Viability of these macrophages was measured by WST-8 assay (C). Mean values are shown with SD. *, P < 0.01. (D) Thioglycollate-elicited peritoneal macrophages from xCT−/− or xCT−/− mice were stimulated with 1 μg/mL of LPS. Forty-eight hours after stimulation, the concentration of HMGB1 in the culture supernatant was measured by ELISA. Mean values are shown with SD. *, P < 0.01.

Results strongly suggest that xCT deficiency causes unremitting inflammation because of impaired survival of activated macrophages at the inflammatory site.

**xCT Deficiency Accelerates Chemically Induced Tumorigenesis.** These findings led us to hypothesize that the absence of xCT may cause serious defects in inflammation-related pathology. Chronic inflammation is closely related to tumor initiation. In addition, macrophages play an important role in inflammation-induced tumorigenesis. Thus, we focused on the possible role of xCT in inflammation-induced tumorigenesis. A single s.c. injection of 3-methylcholanthrene (3-MCA) induced tumors with the characteristics of fibrosarcomas, which develop 2 or 3 months after the injection. Seven days after 3-MCA injection, leukocytes infiltrated the site of injection. Consistent with this, xCT mRNA was significantly up-regulated at the site of injection (Fig. 6A). The crucial role that the microenvironment-derived inflammatory cytokines play in 3-MCA-induced tumorigenesis has been reported [8]. Thus, we examined the expression of IL-1β, IL-6, or TNFα mRNAs at the site of 3-MCA injection in xCT+/+ or xCT−/− mice. IL-1β and TNFα mRNA levels peaked 3 d after injection. As shown in Fig. 6B, IL-1β and TNFα mRNAs were overexpressed 3 d after 3-MCA injection in xCT−/− mice, although IL-6 mRNA levels were not different between WT and xCT−/− mice. These results suggest that xCT deficiency augmented unremitting inflammation. Thus, 3-MCA induced tumorigenesis was assessed
in WT and xCT<sup>mu/mu</sup> mice. As shown in Fig. 6C, early tumor development was observed in xCT<sup>mu/mu</sup> mice; that is, 30% of xCT<sup>mu/mu</sup> mice had a detectable tumor on Day 70, although less than 10% of WT mice carried the tumor on the same day. These results indicate that xCT deficiency causes augmented inflammation at the site of 3-MCA injection and accelerates chemically induced tumorigenesis.

We further examined the growth rate of fibrosarcoma in WT and xCT mutant mice. The day on which tumor was first detected in each mouse was defined as Day 0, and the size of tumors was compared between WT and xCT mutant mice. The growth rate of tumor was not significantly different between WT and xCT mutant mice (Fig. S6A). These results indicate that xCT deficiency only increases tumor incidence, but does not affect growth rate of the tumor once established. We also compared tumor growth using B16 cells, a transplantable melanoma cell line. The tumor growth rate was not different between these mice as similarly observed in fibrosarcoma (Fig. S6B). This result also indicates that xCT does not play an essential role in tumor environment that controls tumor growth.

**Discussion**

Under physiological conditions, xCT expression is generally low in most tissues and cells, and is weakly detected only in brain, thymus, and spleen (25). The present study demonstrated that xCT expression was significantly up-regulated in infiltrating immune cells at the inflammatory site. The transport system xCT<sup>-</sup> is involved in the maintenance of intracellular GSH levels. In this sense, the up-regulation of xCT in macrophages implies an adaptive mechanism against oxidative stress at the inflammatory site. In fact, xCT deficiency impaired the survival of activated macrophages, as described in this study. xCT deficiency affecting the survival of neutrophils has also been reported (26). These findings strongly suggest that excessive cell death of infiltrating cells occurs at the inflammatory sites in xCT-deficient animals. We also examined inflammation-induced myeloid cell proliferation in bone marrow of xCT mutant mice. However, we did not observe any differences in myeloid cell proliferation of bone marrow in response to sterile peritonitis between WT and xCT mutant mice (Fig. S7), suggesting that xCT was not involved in proliferation of myeloid cells in bone marrow.

Recent reports have demonstrated that cell death promotes carcinogenesis. Maeda and colleagues reported that a deficiency of IκB kinase β in hepatocytes augmented hepatocyte cell death by injection of diethylnitrosamine, a chemical hepatocarcinogen (27). The release of intracellular materials from the dying hepatocytes likely activated Kupffer cells to produce inflammatory cytokines and subsequently promoted hepatocarcinogenesis. It is also demonstrated that the hepatocyte-specific transgene of Bcl-2, which possesses antiapoptotic effects, resulted in decreased incidence of liver cancer induced by diethylnitrosamine (28). These findings clearly indicate that excessive cell death and subsequent activation of macrophages result in carcinogenesis. The present study revealed that xCT deficiency accelerated inflammation-induced tumorigenesis. It is most likely that the defect of protection against oxidative stress in inflammatory infiltrating cells resulted in excessive macrophage and neutrophil cell death, and this excessive cell death caused unmitting inflammation that is demonstrated by the overproduction of IL-1β, consequently potentiating tumorigenesis. As far as we know, this work is unique in describing the relationship of impaired survival of macrophages and neutrophils with the acceleration of tumorigenesis. Further analysis should be performed to reveal the relationship between macrophage dysfunction and tumorigenesis.

This study demonstrated that the impaired survival of macrophages induced the release of a large amount of HMGB1, a potent activator of inflammation. Although lacking in direct evidence, it is hypothesized that HMGB1 may be involved in the tumorigenesis in xCT-mutant mice. HMGB1 was recently reported to possess a variety of functions in inflammatory responses. For example, HMGB1 inhibits the phagocytosis of apoptotic neutrophils by macrophages (29). As the clearance of apoptotic neutrophils is required for resolution of inflammation, this effect may be an alternative mechanism by which HMGB1 enhances inflammatory responses. In addition to and consistent with its inflammatory effects, HMGB1 is also involved in tumor initiation and growth (30). Because activated macrophages express a large amount of HMGB1, necrotic cell death of activated macrophages may result in the release of a considerable amount of HMGB1. It is of great interest to examine the potential roles of HMGB1 released from damaged macrophages in tumorigenesis.

Macrophages are involved not only in the initiation of tumorigenesis, but also in the continuous growth of tumors. Tumor-associated macrophages (TAMs) constitute a large portion of the tumor mass and are involved in progression and metastasis of cancer in several ways, including promotion of matrix remodeling, invasion, and angiogenesis (31). TAMs are also reported to possess an immunosuppressive function and are thought to be mainly responsible for immune dysfunction against tumors (32, 33). It is well known that exponential tumor growth results in the generation of a necrotic area within the tumor because of hypoxia. Because cancer cells often contain increased levels of ROS (34), TAMs are possibly exposed to ROS released by the necrotic cancer cells. Thus, we speculated that xCT deficiency might affect TAMs’ survival. However, the number and percentage of tumor-infiltrating myeloid cells and tumor angiogenesis were not different between WT and xCT mutant mice (Fig. S8A and B). In addition, the expression levels of xCT in tumor-associated macrophages were very low (Fig. S8C). These results indicate that TAMs are completely different from activated macrophages infiltrating at the site of inflammation.
This study demonstrated that xCT-deficiency accelerated the initiation of fibrosarcoma induced by 3-MCA. In sharp contrast to the present study, a high expression of xCT gene was demonstrated in some human cancer cells (35), and pharmacological inhibition of system x_{μ} suppressed the proliferation of these cancer cells (36). We found that the expression levels of xCT mRNA were indeed higher than those in normal skin. However, there were no differences in the growth rate of the fibrosarcoma once the tumor was established between WT mice and xCT^{+/-} mice. The fibrosarcoma may have an alternative mechanism to maintain the intracellular cyst(e)ine and GSH levels.

In summary, the defect of the protective system against oxidative stress impaired the survival of activated macrophages and subsequently enhanced tumorigenesis.

Materials and Methods
ENU Mutagenesis, Breeding of Mice, and Screening for Mouse Mutants. ENU mutagenesis was performed as previously described (37, 38). Briefly, ENU (Sigma-Aldrich) was injected intraperitoneally into C57BL/6J Jcl (B6) male mice (Japan CLEA) at 8 to 10 weeks of age with 85-mg/kg body weight. The injections were carried out twice at weekly intervals. The injected male mice were mated with WT B6 female mice by in vitro fertilization pre-embryo transfer procedure after a sterile period (≈10–11 weeks) to produce first-generation (G1) offspring. The G1 male mice were mated again with WT B6 female mice to obtain second-generation (G2) offspring harboring the ENU mutation at 50% of the first generation. Third-generation (G3) offspring were produced by G2 sib-mating. Every reproduction step was accomplished using 85-mg/kg body weight.


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