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Inhibition of xCT effectively induces ferroptosis in oral squamous cell carcinoma cells in epithelial-mesenchymal transition

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Ferroptosis is a newly discovered form of cell death that exhibits effective tumor suppression in various cancer cell types. Since ferroptosis efficiently decreases the viability of cancer cells undergoing epithelial-mesenchymal transition (EMT), it may exert antitumor effects on highly malignant oral squamous cell carcinoma (OSCC) in EMT. xCT is a subunit of a the cystine-glutamate antiporter. Ferroptosis occurs due to increased oxidative stress by inhibiting xCT. We induced EMT in OSCC cells and examined the effects of the induction of ferroptosis by inhibiting xCT in these cells. We investigated whether xCT inhibitor erastin induced ferroptosis in OSCC cells *in vitro.* Furthermore, we induced the EMT state in OSCC cells by the administration of transforming growth factor-β1 and examined the sensitivity of OSCC cells to ferroptosis in the presence or absence of the EMT state. Erastin induced ferroptosis in the OSCC cells. Ferroptosis-induced reductions in cell viability were greater in OSCCs of the mesenchymal state by EMT than in those in the normal state. OSCC cells in the mesenchymal state induced by EMT were highly susceptible to ferroptosis. The mesenchymal properties induced by EMT increase the malignancy of cancer cells. Therefore, therapy involving the induction of ferroptosis has potential in the treatment of advanced OSCC with invasion and metastasis. (J Osaka Dent Univ 2023; 57: 17-24)

Key words: Oral squamous cell carcinoma; Ferroptosis; Epithelial mesenchymal transition; xCT; GPX4

INTRODUCTION

Oral cancer accounts for 2-4% of all cancers worldwide and is the sixth most common malignancy globally.[1] The most common histopathological type of oral cancer is squamous cell carcinoma.[2] Oral squamous cell carcinoma (OSCC) frequently metastasizes to the lymph nodes and/or distant sites. Chemotherapy and radiotherapy are effective for local OSCC, but not for advanced OSCC. Therefore, the development of novel therapies to improve the survival of patients with advanced OSCC is needed. Ferroptosis is a new type of cell death that is induced by the accumulation of lipid reactive oxygen species.[3] It has different characteristics from other forms of cell death, such as apoptosis, necroptosis, and autophagy. Resistance to apoptosis is crucial for the proliferation of tumor cells. Various cancer types including OSCC are characterized by this resistance.[4, 5] Ferroptosis may be useful as an alternative chemotherapy even for cancer cells that resist apoptosis. Therefore, molecular studies have been conducted on the use of agents that induce ferroptosis to treat cancer, in order to elucidate the susceptibility to and the mechanisms underlying ferroptosis in various cancers, such as renal cancer, hepatocellular carcinoma, and breast cancer.[6]

Epithelial-mesenchymal transition (EMT) refers to a molecular transition in which epithelial cells acquire mesenchymal properties for migration.[7] Epi-

thelial cancer cells reorganize the cytoskeleton and resolve cell-to-cell junctions during EMT, which allows for their migration. The activation of EMT enhances tumor malignancy by promoting local tumor invasion, intravascular invasion, and extravasation into the circulation. Therefore, EMT is associated with a poor prognosis and distant metastasis in various human cancers.[8] Although tumor cells during EMT have been shown to resist apoptotic cell death, they are in a state of high oxidative stress, which may increase susceptibility to ferroptosis.[9, 10]

xCT is a subunit of a the cystine-glutamate antiporter (system x_c^-).[11] In the antioxidant stress mechanism, xCT transports cystine from extracellular to intracellular sites, where cystine is used in the synthesis of glutathione. Glutathione peroxidase 4 (GPX4) uses glutathione as a coenzyme to detoxify lipid peroxides into lipid alcohols. These processes play important roles in the control of intracellular oxidative stress, and their inhibition induces ferroptosis in cells due to disruption of the intracellular redox potential by oxidative stress. Erastin may reduce cellular cystine levels by inhibiting the cystine transporter xCT, and this lack of intracellular cystine decreases glutathione levels because the substrate of glutathione synthesis is cystine. Since glutathione is a cofactor required by GPX4 for the degradation of hydroperoxides, the inhibition of cystine uptake by xCT may enhance intracellular oxidative stress.[12, 13] Therefore, ferroptosis is induced in cancer cells by oxidative stress when the activity of GPX4 is reduced.

We investigated the susceptibility to ferroptosis in highly malignant OSCC cells in EMT. The results indicated the utility of ferroptosis-inducing therapy for OSCC that is progressively malignant due to EMT.

MATERIALS AND METHODS

Cell culture

The human OSCC cell lines SAS and HSC-2 were purchased from the Japanese Collection of Research Bioresources (Osaka, Japan). They were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (Bio-West, Nuaillé, France), 100 U/mL penicillin, and 100 μg/mL streptomycin (Nacalai Tesque). Cells were cultured at 37°C in an atmosphere of 5% CO_2 and 100% humidity in an incubator. Erastin (Sigma-Aldrich, St. Louis, MO, USA) and ferrostatin-1 (Fer-1, Cayman Chemical, Ann Arbor, MI, USA) were dissolved in dimethyl sulfoxide (DMSO) (Nacalai Tesque) and stored at −20°C. Culture medium containing the same quantity of DMSO was used as the control.

Cell viability assay

SAS and HSC-2 cells were seeded on 96-well culture plates. In the erastin test, cells were treated with 1, 2, 5, 10 and 20 μM of erastin for 24 h. In the EMT test, cells were treated with/without 10 ng/mL transforming growth factor-β1 (TGF-β1) (PeproTech, Cranbury, NJ, USA). After a 24-h incubation, the cells were washed with fresh medium, and then treated with 0.5, 1, 2, 5 and 10 μM of erastin for 24 h. The control group was treated with DMSO only. Twenty-four hours later, the cell viability was detected by Cell Counting Kit-8 (Dojindo Molecular Technologies, Kumamoto, Japan), according to the manufacturer's instructions.

Flow cytometry

Cells were seeded on 6-mm culture dishes for 24 h and then treated with 5 μM erastin. The control group was treated with DMSO only. Cells were incubated with fresh medium containing 2 μM BODIPY 581/591 C11 dye (Image-iT Lipid Peroxidation Kit) (Invitrogen, Waltham, MA, USA) at 37°C for 20 min, according to the manufacturer's instructions. Cells were harvested by trypsinization and resuspended in PBS. Fluorescence intensity with BODIPY 581/591 C11 staining was analyzed by flow cytometry (FACS Verse; BD Biosciences, Mississauga, ON, Canada).

Western blotting

Cells were seeded on 6-mm culture dishes and treated with 2, 5 and 10 μM of erastin or 10 ng/mL TGF-β1. After 24 h, cells were lysed at 4°C in Cell-

LyEX MP (for the detection of xCT) (TOYO B-Net, Tokyo, Japan) or RIPA lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% Nonidet P 40 Substitute, 0.5% Sodium Deoxycholate: pH 7.6) (Nacalai Tesque). A sample was then subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (Bio-Rad Laboratories, Hercules, CA, USA) and transferred to polyvinylidene difluoride membranes (Bio-Rad). Membranes were blocked with Blocking One (Nacalai Tesque) for 30 minutes, incubated with primary antibodies at room temperature for 1 hour, and then incubated with HRP-conjugated secondary antibodies at room temperature for 30 minutes. The following primary antibodies were used: rabbit monoclonal anti-xCT (CST; #12691) (Cell Signaling Technology, Danvers, MA, USA), rabbit monoclonal anti-GPX4 (ab 125066) (Abcam, Cambridge, England), rabbit monoclonal anti-E-cadherin (CST; #3195), rabbit monoclonal anti-N-cadherin (CST; #13116), rabbit monoclonal anti-vimentin (CST; #5741), rabbit monoclonal anti-slug (CST; #9585) and rabbit monoclonal anti-GAPDH (CST; #2118). Goat anti-rabbit IgG HRP-linked secondary antibodies (CST; #7074) were used. GAPDH was used as a loading control. All antibodies were diluted at 1:1000. Signals were visualized with ImmunoStar Zeta or ImmunoStar LD (Wako, Osaka, Japan) and Chemi-Doc (Bio-Rad).

Reverse transcription polymerase chain reaction

Total RNA was extracted from cells using the RNeasy mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Synthesis of cDNA was performed by reverse transcription using the PrimeScript RT Master Mix (Takara Bio, Kusatsu, Shiga, Japan). The quantification of polymerase chain reaction (PCR) products was performed using the Step One Plus system (Applied Biosystems, Foster City, CA, USA) with TB Green Premix Ex Taq Ⅱ (Takara Bio). The amplification program comprised 40 cycles of denaturation at 95°C for 5 seconds, annealing at 55°C for 20 seconds, and extension at 72°C for 20 seconds. The qPCR results for each sample were normalized to those for GAPDH. Results were expressed as normalized ratios, and all experiments were repeated three times. The primer sequences used were as follows: xCT: forward: 5'-TCTCCAAAGGAGGTTACCTGC-3'; reverse: 5'-AGACTCCCCTCAGTAAAGTGAC-3'; GPX4: forward: 5'-GAAGTAAACTACACTCAGCTC-3'; reverse: 5'-CTCTTTGATCTCTTCGTTACTC-3'; E-cadherin: forward: 5'-TACACTGCCCAGGAGCCAGA-3'; reverse: 5'-TGGCACCAGTGTCCGGATTA-3'; N-cadherin: forward: 5'-CGAATGGATGAAAGACCCATCC-3'; reverse: 5'-GCCACTGCCTTCATAGTCAAACACT-3'; vimentin: forward: 5'-GGTGGACCAGCTAACCAACGA-3'; reverse: 5'-TCAAGGTCAAGACGTGCCAGA-3'; slug: forward: 5'-TGTTGCAGTGAGGGCAAGAA-3'; reverse: 5'-GACCCTGGTTGCTTCAAGGA-3'; GAPDH: forward: 5'-CAGCCTCAAGATCATCAGCA-3'; reverse: 5'-ACAGTCTTCTGGGTGGCAGT-3'.

Statistical analysis

All data are shown as the mean and standard deviation (SD). The Mann-Whitney U test was used for comparisons between two groups. Williams' multiple comparison test was used to compare differences between the control group and experimental group within multiple groups. Scheffé's multiple comparison test was used to compare differences between any two of the groups examined herein. The significance for differences in all tests was set as $*p < 0.05$.

RESULTS

Erastin induces ferroptosis in OSCC cells

Erastin is a compound that specifically induces ferroptosis in human cancer cells.[12] To investigate whether erastin induces ferroptosis in OSCC cells, we analyzed its cytotoxicity. The viability of SAS and HSC-2 cells was significantly lower following the treatments with erastin than in the control group, which was dependent on the concentration of erastin (Fig. 1 A). In addition, Fer-1, an inhibitor of ferroptosis, reduced the viability of erastin-treated SAS and HSC-2 cells (Fig. 1 B). Fer-1 has been shown to reduce the accumulation of lipid reactive oxygen species, thereby preventing ferropto-

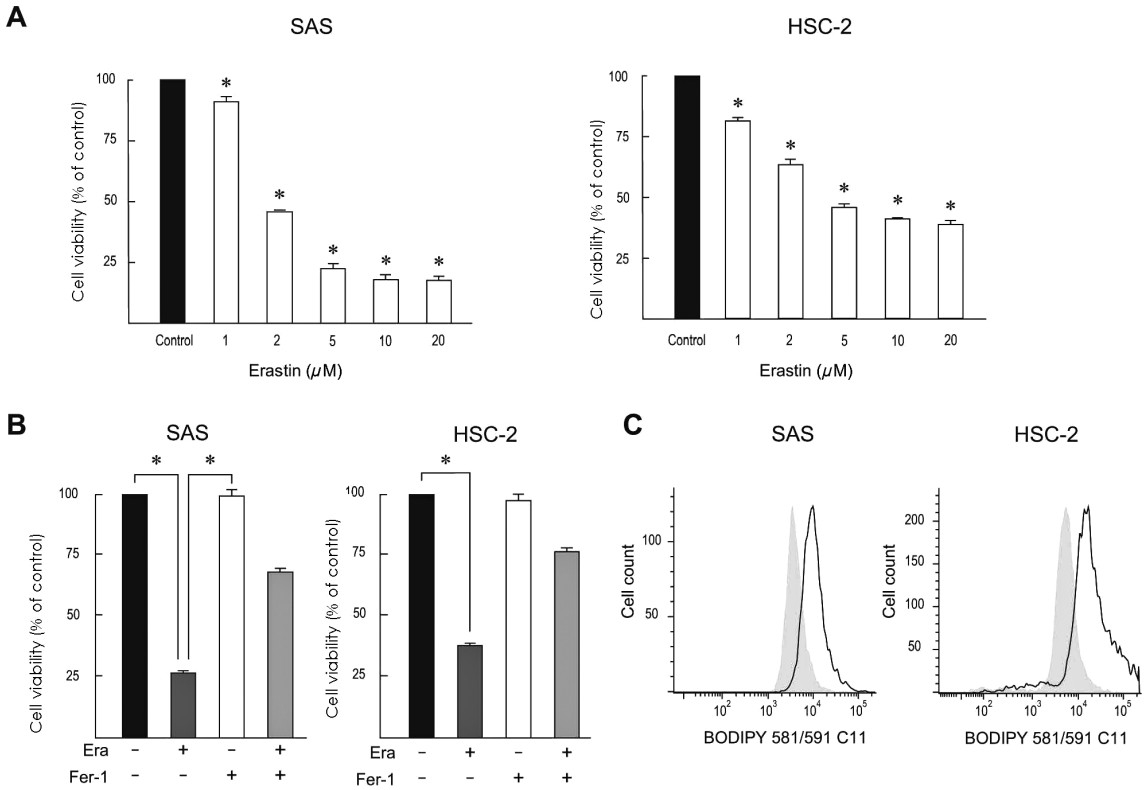

Fig. 1 Erastin induced ferroptosis in OSCC cells. Erastin inhibits the viability of OSCC cells by inducing ferroptosis through the accumulation of lipid peroxides. (A) The viability of SAS and HSC-2 cells treated with 1, 2, 5, 10 and 20 μM of erastin. Data represent the mean and standard deviation of three independent experiments (*$p<0.05$ vs. control). (B) The viability of SAS and HSC-2 cells in the absence or presence of 15 μM erastin (Era) and 20 μM ferrostatin-1 (Fer-1). Data represent the mean and standard deviation of three independent experiments (*$p<0.05$ between the two indicated groups). (C) Detection of lipid peroxides in SAS and HSC-2 cells treated with 5 μM erastin by flow cytometry (black bar), with cells not treated with erastin as the control group (black line).

sis due to oxidative stress.[3] We also demonstrated that lipid peroxide levels in both cell types were higher in the erastin-treated groups than in the controls using flow cytometry (Fig. 1 C). Collectively, these results showed that erastin induced ferroptosis through the accumulation of intracellular lipid peroxides in OSCC cells.

Erastin inhibits activity of GPX4 in OSCC cells

Erastin inhibits xCT and GPX4, which is reported to trigger ferroptosis.[14] Therefore, we investigated whether erastin inhibited the expression of these factors. Western blotting showed that erastin increased the protein expression of xCT in SAS and HSC-2 cells; significantly higher xCT mRNA levels were observed in the erastin-treated groups than in the controls (Figs. 2 A and B), and Western blotting revealed that erastin decreased the expression of GPX4 in both cell types (Figs. 2 C and D). Previous studies indicated that erastin induced the compensatory up-regulation of xCT rather than inhibiting its expression, and may also directly inhibit xCT activity.[12, 15] Our experiments on OSCC cells demonstrated that erastin suppressed the antioxidant mechanism including GPX4 expression by inhibiting the cystine transporter xCT; accordingly, these results supported SAS and HSC-2 cells being in a state of ferroptosis due to oxidative stress through the accumulation of lipid peroxides.

Ferroptosis effectively decreased viability of OSCC cells in EMT by TGF-β1

We then examined sensitivity to ferroptosis in OSCC cells in the mesenchymal state. To confirm

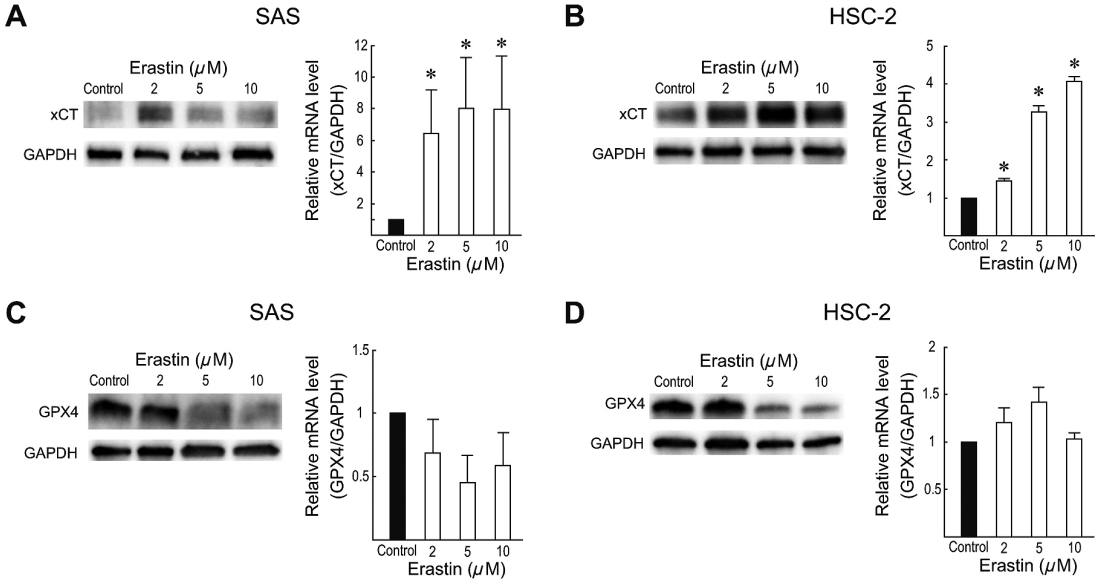

Fig. 2 Erastin decreased GPX4 expression by inhibiting xCT. Erastin increased expression of xCT and decreased GPX4 in OSCC cells. (A-D) The protein expression of xCT and GPX4 in SAS and HSC-2 cells treated with various concentrations of erastin by Western blotting, and the mRNA expression of xCT and GPX4 in SAS and HSC-2 cells treated with various concentrations of erastin by qRT-PCR. GAPDH were used as the standard control in both tests. Data on qRT-PCR represent the mean and standard deviation of three independent experiments (*$p < 0.05$ vs. control).

whether EMT was induced by TGF-β1 in OSCC cells, we measured the expression levels of the TGF-β1-targeting factors E-cadherin, N-cadherin, vimentin, and slug. Western blotting showed that TGF-β1 increased the protein expression of N-cadherin, vimentin, and slug in SAS cells. Vimentin and slug mRNA levels in SAS cells following the administration of TGF-β1 were significantly higher than those in the controls (Fig. 3 A). Similar results were obtained for HSC-2 cells (Fig. 3 B). Vimentin and slug are mesenchymal markers and their expression increases when EMT induces mesenchymal transition.[16] Therefore, we investigated whether erastin-mediated ferroptosis was more strongly induced in OSCC cells in the mesenchymal state. The viability of SAS and HSC-2 cells, which were in the mesenchymal state due to the administration of TGF-β1, were lower following the treatment with each concentration of erastin than in the normal state, with significant variability being observed at some concentrations (Fig. 3 C). Collectively, these results support OSCC cells in the mesenchymal state being more susceptible to ferroptosis than those in the normal state.

DISCUSSION

The present results demonstrated that erastin induced ferroptosis in OSCC cells. The induction of ferroptosis by the inhibition of xCT may be useful in specific anti-cancer chemotherapies because xCT is expressed on many solid malignant tumors, but not on most normal cells.[17] Furthermore, erastin may target mitochondrial resident voltage-dependent anion channel 2 (VDAC2). Erastin has been shown to inhibit the permeability of VDAC2 to the endogenous substrate NADH and, as a result, the oxidation of NADH is reduced, leading to mitochondrial dysfunction and the release of oxidized species.[6] In summary, erastin uses multiple mechanisms to induce ferroptosis in cancer cells. Various compounds besides erastin are being investigated in research on ferroptosis, such as sulfasalazine, sorafenib, and RSL3.[18] These compounds inhibit the degradation of toxins by GPX4. xCT-GPX4-related antioxidant mechanisms in cancer cells have been extensively examined in order to provide

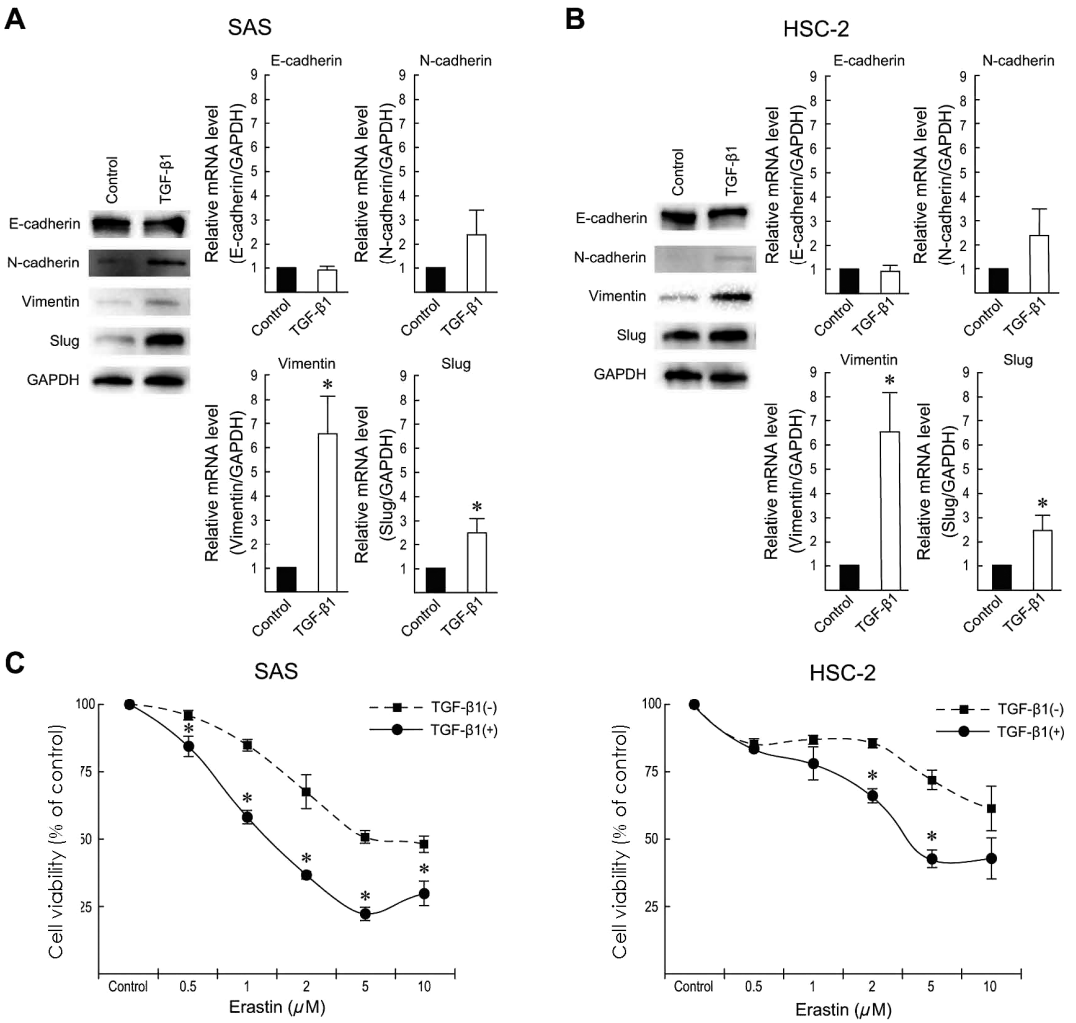

Fig. 3 Decrease in viability of OSCC cells by ferroptosis during EMT. Protein and mRNA expression levels of mesenchymal markers, vimentin and slug were increased in OSCC cells treated with TGF-β1, and OSCC cells in the mesenchymal state induced by EMT were sensitive to erastin-induced ferroptosis. (A, B) EMT-related protein and mRNA expression levels in SAS and HSC-2 cells in the absence or presence of 10 ng/mL TGF-β1. Data represent the mean and standard deviation of three independent experiments (*$p < 0.05$ vs. the control). (C) The viability of SAS and HSC-2 cells treated with 0.5, 1, 2, 5 snd 10 μM of erastin and 10 ng/mL TGF-β1, or without TGF-β1. Data represent the mean and standard deviation of three independent experiments (*$p < 0.05$ vs. the same concentrations in the TGF-β1(−) group).

insights into the control of ferroptosis.

Ferroptosis suppressor protein 1 (FSP1) was recently discovered. It is independently involved in the mechanisms responsible for the prevention of ferroptosis, independent of the antioxidant mechanism based on GPX4.[19, 20] FSP1 acts as a critical enzyme that reduces ubiquinone to ubiquinol. Ubiquinol functions as an antioxidant that counteracts the lethal peroxidation of phospholipids. Therefore, FSP1 prevents the induction of ferroptosis in cancer cells. FSP1 is complementarily promoted even under the functional inhibition of GPX4. Therefore, the inhibition of xCT-GPX4 alone was shown to be insufficient for the complete control of the induction of ferroptosis in cancer cells. iFSP and NPD 4928 have been identified as compounds that control the ferroptosis defense mechanism by FSP1.[21] Limited information is currently available on the effectiveness of FSP1 control, and further research is awaited on its potential effectiveness in

combination with GPX4 control. Therefore, further studies on ferroptosis induced by the inhibition of GPX4 in combination with FSP1 are warranted.

We found that TGF-β1 increased the expression of vimentin and slug, and that OSCC cells in EMT were more susceptible to ferroptosis. EMT involves the transformation of epithelial cells, which adhere to each other via cadherin, into highly mobile mesenchymal cells. Mesenchymal cells secrete large amounts of the extracellular matrix and use their interaction with the matrix to migrate through tissue. In cells that acquired mesenchymal properties by EMT, the level of the epithelial marker E-cadherin was shown to decrease, whereas those of mesenchymal markers, such as N-cadherin, vimentin, snail, and slug, increased.[16] Cancer cell invasion and metastasis may be attributed to the migration of cancer cells that have acquired mesenchymal properties through EMT. Therefore, the induction of ferroptosis in cancer cells in EMT may be useful for the control of advanced OSCC that has undergone invasion and metastasis. The present results demonstrated that OSCC cells in EMT with the expression of vimentin and slug were more susceptible to ferroptosis. EMT also contributes to cancer cell resistance to standard chemotherapy drugs, and the inhibition of EMT has been shown to reduce drug resistance in cancer chemotherapy.[22, 23] Since resistance to anticancer drugs is a major hindrance to cancer control in chemotherapy strategies, the management of EMT cells by ferroptosis is a promising approach for the treatment of various epithelial cancers.

There were two important results in the present study: first, erastin induced ferroptosis in OSCC cells, and secondly, OSCC cells in the mesenchymal state were more sensitive to erastin than those in the epithelial state. These results will contribute to the development of new chemotherapeutic strategies for patients with advanced OSCC. OSCC cells in EMT induced by TGF-β1 were used in the present study. TGF-β1 is an important cytokine that functions in the EMT state, and treatments with TGF-β1 induce EMT in some epithelial cells. A relationship has been reported between increased levels of TGF-β1 and the acquisition of drug resistance in tumors.[10] However, other signaling pathways that cause EMT in cells include the Wnt, Hedgehog, and Notch signaling pathways.[24] EMT in tumor cells has been shown to be promoted by cytokines, such as interleukin-6 secreted by cancer-associated fibroblasts in the tumor microenvironment, as well as by the activation of hypoxia inducible factor 1α under hypoxic conditions.[25, 26] Both of these resulted in the overexpression of the drug efflux transporter multiple drug resistance 1, which contributes to drug excretion. These mechanisms help tumors acquire drug resistance. However, a limitation of the present study is that cases in which EMT was induced by factors other than TGF-β1 were not examined; therefore, further studies are needed to elucidate the susceptibility of cells to ferroptosis by various induction mechanisms of EMT.

CONCLUSION

The present study showed that ferroptosis was induced in the OSCC cell lines SAS and HSC-2 by erastin and that OSCC cells in the mesenchymal state induced by EMT were highly susceptible to erastin-induced ferroptosis. The induction of a mesenchymal state in cancer cells by EMT contributes to the malignant potential of cancer, including invasion and metastasis. Therefore, the present results might contribute to the development of treatment strategies for advanced OSCC using ferroptosis.

This study was conducted with the approval of the Ethics Committee of Osaka Dental University (Approval No. 111040), and all procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and later versions. The authors have no conflicts of interest. This work was supported by the Japan Society for the Promotion of Science (JSPS Grant, KAKENHI, No. JP19K19217).

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