論　文　内　容　要　旨

p53 status modifies cytotoxic activity of lactoferrin under hypoxic conditions

（p53 は低酸素条件下でラクトフェリンの細胞傷害活性を修飾する）

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Lactoferrin (LF) is an 80-kD iron binding glycoprotein of the transferrin family with a wide spectrum of biological effects, including anti-bacterial, anti-inflammatory, immunomodulatory, and anti-cancer activities. LF is found in various fluids secreted from glandular systems, including tears, perspiration, and milk, so it is generally considered to be non-toxic. Bovine lactoferrin (bLF) is now approved as a nutritional product and has been reported to inhibit colon, esophagus, lung, and bladder carcinogenesis in rats when administered orally in the post-initiation stage, suggesting it as a potential therapeutic agent. However, the detailed molecular mechanisms of anti-cancer activity of LF have not been fully determined. Tissue hypoxia is well-known to contribute to biologically aggressive tumor phenotypes and emergence of therapeutic resistance. Hypoxia-inducible factor-1α and -2α (HIF-1α and HIF-2α) are key transcription factors regulating a variety of hypoxia-inducible genes. Hypoxia is also known to induce stress in the tissue microenvironment, which activates p53 signals. Tumor suppressor p53 protein works as a guardian of genome stability, regulator of the cell cycle, and conveyor of cell death signals including apoptosis and ferroptosis. Ferroptosis is a recently identified mode of cell death that is initiated by oxidative perturbations of the intracellular microenvironment and can be inhibited by iron chelators and lipophilic antioxidant. Because LF itself is a natural iron chelator, albeit a more hydrophilic antioxidant, it is expected to modify ferroptosis, especially in cancer cells.

In this study, I undertook to clarify the cytotoxic functions of LF on various cell lines under hypoxic conditions and further understanding of those molecular mechanisms.

As results, inhibitory effects of LF on cell proliferation were observed in cell lines with a wide variety of sensitivities to LF. Firstly, regardless of whether cells were normal or malignant, LF inhibited cell proliferation of normal fibroblast (TIG-3 and KD), squamous

cell carcinoma (HSC2), and hepatoma (HepG2) cells. LF also inhibited proliferation of MCF-7 and HeLa cells with significantly higher IC50 value than other cells, suggesting that cells derived from female-specific organs may have distinct mechanisms to resist cytotoxicity of LF. Interestingly, LF treatments oppositely affected KD and HSC2 under hypoxic conditions: Hypoxia decreased sensitivity to LF in KD but increased sensitivity in HSC2. In order to clarify the molecular mechanisms of differential effects of LF on those cells, cellular characteristics were compared between those two cell types. At first, the effect of LF on levels of HIF-1α and -2α proteins were determined. Hypoxia-stabilized HIF-1α and -2α proteins in KD strikingly increased with LF treatments, but those in HSC2 decreased. LF treatment interestingly decreased DEC2, which is one of the HIF-target genes, in KD but increased that in HSC2, suggesting a possible relationship between LF-modified DEC2 expression and HIF-1α protein level. Secondly, histological differences between KD and HSC2 were considered, since KD is derived from mesenchymal tissue and HSC2 from epithelial tissue. Hypoxia seemed to increase E-cadherin protein in HSC2 and LF treatment slightly decreased it. LF treatment increased vimentin protein level in KD. N-cadherin decreased in hypoxic HSC2 but not in KD, and increased in LF-treated KD but decreased in HSC2. These results indicate that LF treatment enhances mesenchymal phenotype in KD but causes a loss of histological features in HSC2, suggesting that the signal relating to mesenchymal features might be associated with response to LF. Finally, one of the well-known differences between benign (KD) and malignant (HSC2) cell lines was clear: KD has wild-type p53 but HSC2 has mutant-type p53. Protein of p53 was detected in KD but not in HSC2. LF treatment decreased BCL2 expression and slightly increased CDKN1A in HSC2, suggesting inhibition of anti-apoptotic signal and induction of cell cycle arrest in HSC2 under hypoxic conditions. Because LF seemed to regulate

apoptosis and the cell cycle in cells with mutant-type p53, we employed HepG2 expressing mutant-type p53 (MT5). MTT assay strikingly demonstrated that MT5 were significantly more sensitive to LF than control HepG2 (C4), suggesting an important role of the p53 signal in LF sensitivity. Level of BCL2 expression was higher, and levels of BAX and CDKN1A were lower in MT5 than in C4, resulting in phenotypes with anti-apoptotic and progressive cell cycle. In fact, MT5 is more resistant than C4 to the anti-cancer drug bleomycin, and hypoxia drastically decreased sensitivity to bleomycin, but LF treatment sensitized both clones, suggesting potential application as a sensitizer to anti-cancer therapies. The hypoxia-inducible gene DEC2 and the histological marker gene CDH2 were expressed at lower levels in MT5 under both normoxic and hypoxic conditions, suggesting that DEC2 and N-cadherin might contribute to differences between C4 and MT5. Knock-down of TP53 interestingly reduced sensitivity to LF in HepG2 under normoxic and hypoxic conditions, although inhibition of p53 signal was expected to sensitize to LF. This might suggest that p53 signal itself is a target of LF cytotoxic activity, and loss of p53 signal would imply loss of the target of LF, resulting in decreased sensitivity or an alteration of determinants. To further clarify detailed mechanisms of the cytotoxic activity of LF, we focused on ferroptosis. Erastin is a ferroptosis inducer that inhibits the cysteine transporter system, resulting in inhibition of antioxidation by the glutathione peroxidase family. Although Erastin inhibited cell viability of both C4 and MT5, MT5 was significantly more resistant to Erastin. A ferroptosis inhibitor, UAMC-3203, drastically decreased sensitivity to Erastin in both clones under hypoxic conditions, suggesting that hypoxia induces ferroptosis in those cells. In fact, ferroptosis inhibiting SLC7A11 decreased in hypoxic C4 and promoting ACSL4 increased in hypoxic MT5, suggesting that mechanisms of hypoxia-induced ferroptosis are different in those clones. Importantly, LF treatment increased

ACSL4 only in hypoxic MT5, suggesting the existence of LF-induced ferroptosis in cells expressing mutant-type p53.

In conclusion, hypoxia was found to regulate LF sensitivity differently among different cell lines, possibly through the p53 signaling pathway including DEC2 and N-cadherin. LF was further suggested to regulate ferroptosis through expression of ACSL4, depending on p53 status. This study provides novel insight into the clinical application of lactoferrin.