主論文の要旨

Secretory expression of mammalian NOTCH tandem epidermal growth factor-like repeats based on increased O-glycosylation

O−グリコシル化の増加に基づく哺乳類NOTCHタンデム
上皮成長因子様リピートの分泌発現

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【**Introduction**】

Dysregulation of Notch signaling is associated with various cancer. Although Notch signaling has both tumor-promoting and tumor-suppressive roles, it is considered a potential therapeutic target for tumors because tumor angiogenesis depends on Notch signaling; disruption of Notch signaling inhibits tumor growth by impairing angiogenesis. Thus, establishing effective strategies to inhibit Notch signaling is of therapeutic importance. A previous study developed a soluble form of the NOTCH1 receptor (NOTCH1 decoy) that inhibits ligand-induced Notch signaling and tumor angiogenesis in vivo. Although the NOTCH1 decoy molecule consists exclusively of a tandem array of epidermal growth factor-like (EGF) repeats, the expressed proteins are poorly secreted into the culture media and are predominantly retained within the cells. NOTCH EGF repeats are modified with specific O-glycans, including O-fucose, O-glucose, and O-GlcNAc, which are catalyzed by POFUT1, POGLUT1, and EOGT, respectively. Previous studies have revealed that POFUT1 and POGLUT1 affect the cell surface expression of endogenous NOTCH1 in various cell types, including HEK293T cells. Thus, optimal expression of EGF domain-specific glycosyltransferases may improve the production of full-length NOTCH EGF repeats.

In this study, we evaluated the effects of overexpressed EGF domain-specific O-glycosyltransferases, including POFUT1, POGLUT1, and EOGT, on the secretion of NOTCH EGF repeats in HEK293T cells. Our results reveal a novel function of EOGT in promoting the secretion of recombinantly expressed NOTCH1 EGF repeats.

【**Method**】

We exogenously expressed EOGT in HEK293T cells to prepare a culture medium containing NOTCH1 EGF repeats. The plasmid construct encoding the entire NOTCH1 EGF repeat with an N-terminal FLAG tag (FLAG:N1-EGF) was generated and transfected into HEK293T cells with or without EOGT. Culture media containing FLAG:N1-EGF were incubated with DLL4 ligand-expressing cells and analyzed by FACS. Next, we examined the combined effects of glycosyltransferases acting on NOTCH EGF domains in the endoplasmic reticulum (ER), including POFUT1, POGLUT1, and EOGT. Because a previous study on *Drosophila* POFUT1 homologue indicated that glycosyltransferases could promote NOTCH secretion in an enzyme activity-independent manner, we analyzed the impact of enzymatically inactive mutants of EOGT and POGLUT1. To evaluate changes in the O-glycosylation states of EGF repeats mediated by the co-expression of EOGT and POGLUT1, mass spectrometric analysis was performed. We selected FLAG-tagged NOTCH3 EGF repeats (FLAG:N3-EGF) for analysis because of the abundance of the purified proteins.

【**Result**】

First, we confirmed that with EOGT overexpression the amount of FLAG:N1-EGF bound to

ligand-expressing cells was significantly increased. At the same time, the amount of FLAG:N1-EGF in the culture media was elevated. These results suggest that EOGT possesses a novel but seemingly physiologically irrelevant ability to promote the secretion of EGF repeats of NOTCH1, which at least partially contributes to increased binding to DLL4. Next, we prepared culture media and cell lysates to analyze the amount of FLAG:N1-EGF, FLAG:N3-EGF secretion. Immunoblotting results indicated that FLAG:N1-EGF secreted in the culture media was increased by co-expression of EOGT. Despite the roles of POFUT1 and POGLUT1 in the secretory pathway, the effect of POFUT1 or POGLUT1 overexpression on FLAG:N1-EGF NOTCH1 secretion was not statistically significant in HEK293T cells. In contrast, co-expression of EOGT/POGLUT1, but not EOGT/POFUT1, further increased secretion compared with EOGT alone. Also, FLAG:N3-EGF secreted in culture media was increased by co-expression of EOGT/POGLUT1. Quantification of secreted FLAG:N1-EGF from *EOGT* mutant HEK293T cells co-expressing inactive EOGT harboring the R377Q mutation showed that EOGT^{R377Q} failed to facilitate secretion into the culture media. Co-expression of POGLUT1^{R279W} slightly increased the secretion of FLAG:N1-EGF, but to a lesser extent than that of wild-type POGLUT1. These data suggested that enzyme activity is essential for the effect of EOGT on the promotion of FLAG:N1-EGF secretion. The optimal combination of ER-resident glycosyltransferases serves as a strategy for efficiently producing EGF repeats of various NOTCH family members based on the engineered expression of glycosyltransferases. Finally, we adopted the label-free quantitation workflow in Proteome Discoverer with Byonic node, a search engine suitable for glycopeptide identification from MS/MS spectra. Consistent with the O-GlcNAc transferase activity of EOGT, the abundance ratio of HexNAc-modified glycopeptides corresponding to O-GlcNAc, O-GlcNAc-Gal, or O-GlcNAc-Gal-NeuAc glycoforms was markedly increased at EGF9, 10, 25, 31, and 34 by EOGT/POGLUT1 overexpression. In contrast, the effect on EGF4, 14, 18, and 30 was limited because O-GlcNAcylation occurs at high stoichiometries regardless of the exogenously expressed glycosyltransferases. These data support our proposal that elevated O-GlcNAc modifications promote the secretion of NOTCH EGF repeats. Lack of these constitutive modifications may contribute to the reduced secretion efficiency of NOTCH EGF repeats in EOGT mutant cells. Unlike in the case of O-GlcNAc modification, the effect of EOGT/POGLUT1 on O-Glc modification was obscure because all detectable O-Glc sites on FLAG:N3-EGF were mostly O-glycosylated without EOGT/POGLUT1 overexpression. However, that NOTCH EGF repeats with insufficient O-glycosylation are not secreted and, therefore, not detected by mass spectrometry of secreted proteins in the culture medium. Expression of POGLUT1 may reduce the proportion of insufficiently O-glucosylated NOTCH EGF, which is otherwise retained in the cells.

【Conclusion】

This study suggests that combining ER-resident EGF domain-specific glycosyltransferases

improved the amount of secreted NOTCH EGF repeats in the culture media. Notably, we showed for the first time that EOGT has the potential to promote the secretory expression of NOTCH EGF repeats. We speculated that overexpressing these glycosyltransferases is effective in the sensitized condition where NOTCH EGF repeats are prone to be under-O-glycosylated. The altered O-glycosylation states of NOTCH EGF repeats were evaluated by mass spectrometry using the simplified label-free quantitation workflow of Proteome Discoverer. These strategies are applicable to the production and quality control of full-length NOTCH EGF repeats for use as pharmaceuticals effective against diseases, including various cancers.