主論文の要旨

Binding of a sialic acid-recognizing lectin Siglec-9 modulates adhesion dynamics of cancer cells via calpain-mediated protein degradation

（シアル酸認識レクチン Siglec-9 の結合がカルパインによる
タンパク質分解を介して癌細胞の接着動態を制御する）

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Aim of this study:

Sialic acids are present as terminal monosaccharides linked to cell surface glycoconjugates, and play roles in a variety of physiological and pathological events. Sialic acid-binding immunoglobulin-like lectins (Siglecs) are unique endogenous lectins recognizing sialic acid-containing carbohydrates on glycoproteins and glycosphingolipids. Siglecs have been thought to regulate the functions of innate immune cells and adaptive immune systems based on glycan recognition. In particular, Siglec-7-mediated attenuation of NK and dendritic cell functions has been reported as an example of escape mechanisms of cancer cells from immune surveillance.

Siglec-9 expressed in monocytes and granulocytes. Although inhibitory functions of Siglec-9 in immune cells via ITIM motifs have been reported in some experimental systems, outcome of the interaction between Siglecs and their sialylated ligands in the side of cells expressing sialyl-compounds has scarcely been studied to date. During the analysis of effects of interaction between Siglec-9 and its counter ligands on cancer cells, we found marked degradation of focal adhesion kinase (FAK) in cancer cells, and also cell detachment from plates. Then, we have analyzed implication of Siglec-9-mediated signaling in the regulation of adhesion dynamics of cancer cells.

Experimental procedures:

Flow cytometry and cell sorting: Cell surface expression of Siglec-9Fc-binding ligands was analyzed by FACS Caliver. Seventy five human cancer cell lines were screened for binding of Siglec-9Fc fusion proteins. To analyze whether sialic acids are essential for Siglec-9Fc binding, cells was incubated with neuramidase. Then, Siglec-9 binding was examined by flow cytometry. To prepare Siglec-9 high-expressing and low-expressing U937 cells, $U937^{siglec\text{-}9\text{-}high}$ cells were sorted from U937 transfected with pcDNA3.1-Siglec-9. $U937^{siglec\text{-}9\text{-}low}$ cells were sorted from $U937^{mock}$ cells by using FACSAria II.

Productions of Siglec-9-Fc fusion proteins: pEE14-Siglec9-3C-Fc plasmid was provided by P.R. Crocker. Siglec-9Fc and Fc secreted from HEK293T cells were prepared by DEAE-dextran transfection, and fusion proteins were affinity purified by Protein A Sepharose.

Real time cell electronic sensing (RT-CES) test: Cell adhesion and growth was dynamically monitored as cell index (CI) using the RT-CES system. AS cells were seeded into 16-well e-plates, then co-cultured with living $U937^{siglec\text{-}9\text{-}high}$. For the stimulation with Siglec-9Fc or Fc, Siglec-9Fc or Fc proteins were applied into e-plates. To examine whether degradation of FAK was caused by calpain, cells were pre-incubated with MDL-28170 (Calpain inhibitor III).

Co-culture experiments: They were performed by adding $U937^{siglec\text{-}9\text{-}high}$ and $U937^{siglec\text{-}9\text{-}low}$ cells to AS cells in 6-well plates, and being co-cultured.

Wound healing assay: Migration activity of AS cells co-cultured with $U937^{siglec\text{-}9\text{-}high}$ or $U937^{siglec\text{-}9\text{-}low}$ was assessed by scratching assay.

Cell migration assay: It was performed with Boyden chamber invasion assay. Then, cell number was counted by ImageJ (v1.47d) software.

Immunoblotting: To examine signaling pathways in AS and other cell lines, cell lysates were analyzed by standard immunoblotting methods. Bands were visualized with an ECL detection system. Band intensities were analyzed with Image J and standardized with β-actin.

Results:

In this study, we found that an astrocytoma cell line, AS showed detachment from culture plates when co-cultured with Siglec-9-expressing cells or soluble Siglec-9 (Fig-1, 2A, 2B and 2C). Moreover, detached AS cells re-grew as co-cultured cells with Siglec-9-deficient cells. FAK underwent rapid degradation in cancer cells during the co-culture (Fig-3A). Other signaling molecules such as Akt, paxillin and p130Cas were also degraded immediately after the co-culture (Fig-3B, C). On the other hand, they showed increased motility and invasiveness upon Siglec-9 binding (Fig-2D, E). These paradoxical results might indicate the differences in the roles of FAK depending on the spatial and temporal dynamics of cell adhesion. Indeed, sustained levels of individual phosphorylated forms were observed during co-culture, suggesting partially overlapping activation of those molecules with FAK degradation (Fig-4).

Among molecules that showed sustained activation levels, we focused on p-Akt T308 because of its increased phosphorylation in the early phase of co-culture (Fig-3B), and could show its localization in the front side of AS cells as well as remaining FAK (Fig-7). These results should explain, at least partly, mechanisms for the increased migration of AS cells undergoing FAK degradation. Increased activation of Akt T308 might be also involved in the resistance to apoptosis in AS.

Discussion:

Results obtained in this study suggested that proteolytic enzymes should be activated and exert for the transient degradation of a wide range of signaling molecules. Possible protease responsible for the degradation of FAK etc should be calpain as revealed by its inhibitor (Fig-5, Fig-6). Results of experiments for mobility and apoptosis also support the concept that the protein degradation observed in co-culture might be beneficial for cancer cell migration and survival in addition to the simultaneously induced inhibitory signals in immune cells (U937 in this study), leading to the escape of cancer cells from immunosurveillance.

Conclusion:

Our results suggest that protein degradation of FAK and related molecules was induced by Siglec-9 binding to its counter receptors via sialylglycoconjugates, and activation of calpain by Siglec-9 and subsequent protein degradation increases cell motility and survival of cancer cells, leading to the modulation of adhesion kinetics of cancer cells. These results might explain a mechanism by which cancer cells utilize Siglec-9-derived signals to escape from immunosurveillance (Fig-8).