博士論文（要約）

Biochemical analysis for cell adhesion mechanism by LI-cadherin

（LI-cadherin による細胞接着機構に関する生化学的解析）

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Chapter 1. Introduction

1.1. Liver Intestine-cadherin

Liver Intestine-cadherin (LI-cadherin) is a member of the cadherin superfamily. Cadherins are a family of glycoproteins responsible for calcium ion-dependent cell adhesion (1). More than 100 types of proteins belong to the cadherin superfamily. The members are classified into six subfamilies and other members based on the domain composition of extracellular cadherin (EC) repeats, protein sequences, and genomic structure (2). Generally, cadherins are composed of several EC repeats, a single transmembrane domain, and a cytoplasmic domain (2). In most cases, three calcium ions bind to the linker between each EC repeat and contribute to the rigidity of the extracellular region (3). Many members of the cadherin superfamily are responsible not only for cell adhesion, but are also related to tumorigenesis or cancer progression, and thus have been intriguing targets for research in molecular biology (4).

Classical cadherins are major members of the cadherin superfamily. They are composed of five EC repeats, a single transmembrane domain and a cytoplasmic domain (2). Generally, three calcium ions are considered to bind to the linker between each EC repeat (5–7). Classical cadherins mediate homodimerization, a fundamental event in cell adhesion (8), by forming a homodimer via interactions between their two N-terminal EC repeats, EC1 and EC2. They first form an intermediate “X-dimer” and then a “strand swap-dimer” (ss-dimer), which is the final form of the homodimer (3, 9–12). The two molecules of the X-dimer interact through the regions

around the linker connecting EC1 and EC2 (11). The ss-dimer is formed by swapping the N-terminal strands of the EC1s between the two components of the dimer. A critical feature of this process is the insertion of a tryptophan (Trp) residue (located at position 2) of the first protomer into the hydrophobic pocket of the partner protomer (9, 10). Classical cadherins also possess a conserved cytoplasmic domain of more than 100 amino acids and the interaction of this domain with catenins is necessary for efficient cell adhesion via classical cadherins (13, 14). Cell adhesion by classical cadherins is believed to be achieved by lateral clustering (cis-interaction) of homodimers formed between the same type of cadherins expressed on adjacent cells (6). The interface of the cis-interaction was inferred from analyses of the crystal lattice (6, 7). A previous study showed that the disruption of the cis-interface in E-cadherin, which is a classical cadherin, abolishes the ordered layer of E-cadherin molecules on liposomes, suggesting that the cis-interface is necessary for the formation of adhesive junctions (7).

LI-cadherin exhibits distinct structural features compared to other members of the cadherin superfamily. LI-cadherin possesses seven EC repeats, single transmembrane domain, and a cytoplasmic domain. Only kidney-specific (Ksp)-cadherin shares structural features with LI-cadherin. Neither of these cadherins belong to any of the previously reported subfamilies (2) and they have thus been termed 7D-cadherin (15). LI-cadherin expression was first identified in rat liver and intestinal cells. Considering its expression sites and its sequence similarity with

classical cadherins (E-, N-, and P-cadherin), the newly identified protein was named “Liver Intestine”-cadherin (16). LI-cadherin lacks a conserved calcium ion-binding motif between the second and third EC repeats (EC2 and EC3, respectively) (17). The cytoplasmic domain of LI-cadherin is composed of approximately 20 amino acids, and the catenin-binding site present in the cytoplasmic domain of classical cadherins is not conserved in LI-cadherin (16). In fact, the LI-cadherin cytoplasmic domain does not require any interaction with other proteins to exhibit a Ca^{2+}-dependent cell adhesion function (18). Interestingly, sequence analysis comparing mouse LI-cadherin with E-, N-, and P-cadherins revealed sequence homology between the EC1-2 domains of these proteins, as well as between EC3-7 of LI-cadherin and EC1-5 of the E-, N-, and P-cadherins (17). The authors of this study suggested that classical cadherins originated from a five-domain precursor cadherin and that the two additional domains of LI-cadherin were obtained through a gene duplication event (17). Nevertheless, the molecular basis of the cell adhesion function of LI-cadherin, including the mechanisms mediating homodimerization, is poorly understood. The major differences between LI-cadherin and classical cadherins are summarized in Table 1.

Table 1. Major differences between LI-cadherin and classical cadherins

	Classical cadherins	LI-cadherin
Number of EC repeats	5	7
Ca^{2+}-binding motifs	Every linker between each EC repeat has the motif	Linker between EC2 and EC3 lacks the motif
Cytoplasmic domain	More than 100 amino acids Interaction with catenin is necessary for cell adhesion	~20 amino acids Interaction with cytoplasmic protein is not necessary for cell adhesion
Homodimerization mechanism	Intermediate: X-dimer Final state: ss-dimer	Unknown

1.2. Expression of LI-cadherin on cancer cells

In the human body, expression of LI-cadherin is observed in normal small intestine and colon cells (19), as well as in various cancer cells, such as gastric adenocarcinoma, colorectal cancer, and pancreatic cancer (19–22). The role of LI-cadherin in cancer cells has been discussed in several publications. A study using LoVo cells (human colorectal adenocarcinoma cells) showed that knockdown of the LI-cadherin gene increased the invasion and metastatic potency of LoVo cells (23). In contrast, knockdown or knockout of the LI-cadherin gene in Panc02-H7 cells (mouse pancreatic ductal adenocarcinoma cells) suppressed cell proliferation *in vitro* and orthotopic tumor growth *in vivo* (22). These results indicated that LI-cadherin may have both positive and negative effects on cancer progression. In the case of gastric cancer, LI-cadherin is considered promising for imaging because its expression is not observed in normal gastric cells but is expressed on gastric cancer cells at a higher rate than other gastric cancer markers, such as CDX2 and CK20 (19, 24).

For these reasons, LI-cadherin has been focused as a target for cancer therapy and diagnosis. However, the molecular mechanisms underlying the contribution of LI-cadherin to cancer growth or suppression remain elusive.

1.3. Potency of LI-cadherin as a target for cancer treatment and diagnosis

As described above, LI-cadherin contributes both positively and negatively to cancer progression and is a promising target for the imaging of gastric cancer. Therefore, multiple approaches are conceivable when targeting LI-cadherin for cancer treatment or diagnosis.

In cancer cells in which LI-cadherin contributes to tumor growth, an inhibitor of LI-cadherin function (an antagonist) may act as an anticancer drug. In contrast, in cancer cells in which LI-cadherin suppresses cancer metastasis, an enhancer of LI-cadherin function (an agonist) may prevent metastasis. Molecules that can stably and specifically bind to LI-cadherin expressed on cancer cells are good candidates for cancer imaging. However, a poor understanding of the molecular basis of LI-cadherin function is an obstacle to designing molecules that exhibit the desired properties.

Indeed, a study which reported the mechanism through which the anti-P-cadherin antibody TSP7 inhibits P-cadherin-dependent cell adhesion relied on existing knowledge of the molecular basis of P-cadherin homodimerization and cell adhesion (25). Furthermore, the authors improved the potency of TSP7 through rational design of the mutation based on an understanding

of the homodimerization mechanism. This study demonstrated the importance of understanding the molecular basis of cadherin function when designing molecules targeting cadherin, and also suggested the potency of antibodies as inhibitors of cadherin function.

1.4. Purpose of the study

In this study, I aimed to elucidate the mechanisms of LI-cadherin-dependent cell adhesion at the molecular level, with the purpose of developing therapeutic and diagnostic molecules targeting LI-cadherin. I expressed LI-cadherin as a recombinant protein and used various biochemical techniques and computational approaches to analyze the mechanisms underpinning its homodimerization and structural features. In addition, I investigated the effect of anti-LI-cadherin antibodies on LI-cadherin-dependent cell adhesion, to evaluate the possibility of using such antibodies for the imaging and treatment of cancers expressing LI-cadherin. In particular, the knowledge obtained from the analysis of the cell adhesion mechanism influenced my investigations of the antagonistic or agonistic activity of each antibody in LI-cadherin-dependent cell adhesion. This information was also applied to elucidate the roles of LI-cadherin in cancer cells at the molecular level. It has been reported that patients with colorectal carcinoma carrying single nucleotide polymorphisms (SNPs) in the LI-cadherin gene have a higher risk of lymph node metastasis (26). I therefore focused on the amino acid changes caused by these SNPs (26) and aimed to elucidate how these changes influence the molecular mechanisms of LI-

cadherin-dependent cell adhesion to increase the risk of cancer metastasis.

In this doctoral thesis, an analysis of the homodimerization mechanism and structural features of LI-cadherin is described in Chapter 2. In Chapter 3, analyses of anti-LI-cadherin antibodies are described, and the mechanisms underlying the inhibition or enhancement of LI-cadherin-dependent cell aggregation is discussed in combination with the results shown in Chapter 2. In Chapter 4, the molecular mechanisms by which SNPs in the LI-cadherin coding gene affect lymph node metastasis in colorectal cancer are described. A summary of the findings and future perspectives of my doctoral thesis are provided in Chapter 5.

Chapter 2.

Dimerization mechanism and structural features of human LI-cadherin

First, LI-cadherin was expressed as recombinant protein. Measurement of the dissociation constant (K_D) using analytical ultracentrifugation (AUC) revealed that LI-cadherin forms two types of homodimer: EC1-4 homodimer and EC1-2 homodimer. Crystal structure of LI-cadherin homodimer revealed the novel architecture of LI-cadherin EC1-4 homodimer, which is different from other cadherins' homodimer. Biochemical and computational analysis were performed to elucidate the detailed characteristics of the EC1-4 homodimer. Molecular dynamics simulation indicated that noncanonical Ca^{2+}-free linker between EC2 and EC3 is highly flexible when homodimer is not formed. Phe224 was identified as a critical residue for the homodimerization of EC1-4 through the analysis using size exclusion chromatography-multi angle light scattering (SEC-MALS) and differential scanning calorimetry (DSC). Cell-based assay has proved that Phe224 is also indispensable for LI-cadherin-dependent cell adhesion. Cell-based assays using truncated LI-cadherin suggested that formation of EC1-2 homodimer is not sufficient to maintain LI-cadherin-dependent cell adhesion.

These results indicated the unique molecular characteristics of LI-cadherin and suggested that LI-cadherin plays unique roles in human body.

Chapter 3.

Analysis of how anti-LI-cadherin antibodies affect LI-cadherin-dependent cell adhesion

Epitope of each antibody was analyzed using hydrogen deuterium exchange mass spectrometry (HDX-MS) and isothermal titration calorimetry (ITC). Some types of IgG and Fab exhibited inhibitory effect on LI-cadherin-dependent cell adhesion whereas some others exhibited enhancement effect. The underlying mechanisms of inhibitory or enhancement activity of each antibody against LI-cadherin-dependent cell adhesion was described based on the knowledge obtained from the analysis on cell adhesion mechanism.

Although further investigation using clinical samples and *in vivo* experiments are necessary to validate the potency of each antibody, my study demonstrates the possibility of regulating LI-cadherin function and performing stable imaging of cancer cells using anti-LI-cadherin antibodies.

Data included in this chapter will be published in 5 years.

Chapter 4.

Molecular mechanism underlying the increased risk of colorectal cancer metastasis due to LI-cadherin gene single nucleotide polymorphisms (SNPs)

Two single nucleotide polymorphisms (SNPs) were found in the LI-cadherin gene (26). The SNPs are responsible for the amino acid changes of Lys115 to Glu and Glu739 to Ala (26).

Homodimerization tendency of both EC1-4 and EC1-2 homodimer were decreased by the mutation of Lys115 to Glu. MD simulation revealed the partial conformation change of the molecule by the mutation of Lys115 to Glu. Cell-based assay showed the decrease of cell adhesion ability by the amino acid changes caused by the SNPs.

The results indicated that the amino acid change by SNPs decrease the homodimerization tendency of LI-cadherin, which is necessary for LI-cadherin-dependent cell adhesion. The risk of cancer metastasis seems to be increased by the weakened cell adhesion ability which enhances the migration of cancer cells from primary tumor. This study is the first to consider the role of LI-cadherin in cancer cells at the molecular level.

Data included in this chapter will be published in 1 year.

Chapter 5. Summary

As little was known regarding LI-cadherin at the molecular level prior to this study, I believe that the knowledge obtained from my research will advance our understanding of the roles of LI-cadherin in both normal and cancer cells, and will aid in the development of therapeutic and diagnostic molecules targeting LI-cadherin.

As future perspectives, I expect that analysis using additional anti-LI-cadherin antibodies would contribute not only to increasing the number of candidates with potential for clinical application, but would also deepen our understanding of the association state of LI-cadherin on the cell membrane.

Data included in this chapter will be published in 5 years.

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