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ORIGINAL ARTICLE

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T-cadherin (CDH13, H-cadherin) expression downregulated surfactant protein D in bronchioloalveolar cells

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Abstract T cadherin is a unique cadherin cell adhesion molecule that is anchored to the surface membrane through a glycosyl phosphatidyl inositol (GPI) moiety. In the present study, we postulated that T cadherin could regulate surfactant protein (SP)-D gene expression in human bronchioloalveolar type-II cells. We transfected A549 cells (human lung cancer cell line with alveolar type-II cell characteristics) with the T-cadherin expression vector. Both original and control plasmid-transfected A549 cells expressed SP-D; however, neither human nor murine T-cadherin-transfected A549 cells expressed SP-D mRNA. The downregulation of SP-D production in human T-cadherin-expressed A549 cells was also demonstrated using Western immunoblotting techniques. Control vector-transfected A549 cells showed a positive band of SP-D but not of T cadherin. In contrast, T-cadherin-transfected A549 cells, which expressed T-cadherin protein, did not produce SP-D. We further examined the relationship of T cadherin and SP-D expression in secondary pulmonary alveolar proteinosis associated with hematolymphoid malignancies. SP-D was detected in bronchioloalveolar type-II cells in alveolar proteinosis. However, little or no T-cadherin expression was detected in alveolar type-II cells in these patients. To our knowledge, this is the first report describing an effect of cadherin on SP production in bronchioloalveolar cells.

Keywords T-cadherin · H-cadherin · Surfactant protein D · Bronchioloalveolar cells

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Introduction

Recent study clearly revealed that human CDH13, identical to H cadherin, is a human homologue of chicken T cadherin. T cadherin was first identified in chicken as a unique glycosyl phosphatidyl inositol (GPI)-linked membrane cadherin [14]. Human CDH13, cadherin13, was subsequently reported by Tanihara and Suzuki et al. [19]. More recently, Lee identified a candidate gene of which expression is altered in human breast cancer [8]. This candidate gene, which was strongly expressed in heart, was termed H cadherin and appeared to be identical to CDH13. The CDH13 gene was mapped on 16q24, in which loss of heterozygosity in patients with sporadic liver, prostate, breast, and lung cancer were reported. [3, 16, 24, 25] Consistent with these findings, Sato et al. reported that the CDH13 gene is inactivated in a considerable number of human lung cancer specimens [17]. They reported that hypermethylation related to inactivation of the T-cadherin molecule was found in 45% of primary lung cancers.

In the present study, we found that an A549 cell line [10], which is well known as a human bronchioloalveolar carcinoma cell line with properties of type-II alveolar cells, did not express T cadherin. In contrast, preliminary immunostaining with anti-T-cadherin antibody demonstrated a focal positive signal in human bronchioloalveolar type-II cells. Therefore, we expected that the transfection of A549 cells with a T-cadherin expression vector may unravel the potential and physiological role of T cadherin in bronchioloalveolar type-II cells. In the present study, we also examined whether T-cadherin expression was associated with surfactant protein (SP) expression in alveolar proteinosis, because previous reports described that the serum concentration of SP-D is increased in patients with alveolar proteinosis [5].

Materials and methods

Cells and antibodies

The A549 cells used in the present study were purchased from the Japan Health Science Research Resources Bank (Osaka, Japan).

Table 1 Primers used in the present study. *SP* surfactant protein

SP-A	Sense	5′>TTTCTTGGAGCCTGAAAAGA>3′
	Antisense	5′>GGAGGCCGAAGGCCAGAGAGCGT>3′
SP-B	Sense	5′>CATCGACTACTTCCAGAACCAGAC>3′
	Antisense	5′>GCAGATTGCCGCCCGCCACCAGAGG>3′
SP-C	Sense	5′>ATGGATGTGGGCAGCAAAGAGGT>3′
	Antisense	5′>AGATGTAGTAGAGCGGCACCTC>3′
SP-D	Sense	5′>TTTGCCAGGAGCTGCAGGGCAA>3′
	Antisense	5′>AAGTGCTCGCAGACCACAAGACG>3′
T cadherin	Sense	5′>GCCACGATCATGATCGATGAC>3′
	Antisense	5′>GTCTTCATTTTCCACTTTGA>3′
G3PDH	Sense	5′>TCCACCACCCTGTTGCTGTA>3′
	Antisense	5′>ACCACAGTCCATGCCATCAC>3′

The cell line was maintained with Dulbecco's medium (Life Technologies, Grand Island, N.Y.) containing 10% heat-inactivated fetal calf serum (FCS) and 50 µg/ml gentamycin (Life Technologies). In the present study, we used both murine- and rabbit-specific antibodies to human T cadherin. The detailed procedure of generating and characterizing these antibodies was previously described [22, 23]. Goat polyclonal antibody to human SP-D was purchased from Santa Cruz Biotechnology Inc, (Santa Cruz, Calif.). We also used isotype-matched monoclonal murine immunoglobulin (Ig)M, which was generated against *Trichosporon beigelli* and was revealed as not being reactive with various human tissues tested [20]. *Trichosporon beigelli* is a fungus. Normal rabbit IgG and normal goat IgG were also used as control antibodies.

Patients and tissue specimens

Specimens were obtained from four patients with secondary alveolar proteinosis. These patients suffered from acute lymphocytic or myelocytic leukemia, and lung specimens were obtained at autopsy. Tumor-free regions of lung tissue from patients with lung cancer were also used in this study.

Immunohistochemical staining and Western immunoblotting

Details of the procedures were previously described [21]. Briefly, tissues were routinely formalin-fixed and embedded in paraffin. The tissue sections were cut, deparaffinized, and immunostained with antibodies using a labeled streptavidin–biotin complex peroxidase kit (Dako LSAB kit; Dakopatts, Tokyo, Japan). Finally, the reaction was developed with diaminobenzidine. The tissue sections were counter stained with hematoxylin or methyl green.

Western immunoblotting was carried out as previously reported [23]. Briefly, control vector- and T-cadherin expression vector-transfected A549 cells were solubilized with lysis buffer [20 mM Tris-HCl, 150 mM NaCl, 0.5% NP-40, 0.01% sodium dodecyl sulfate (SDS), 0.5 mM leupeptin, 0.03 mM aprotinin, pH 7.4]. The protein concentration was quantified using the method of Bradford (Bio-Rad, Hercules, Calif.), and equal protein amounts were used for electrophoresis. We also used protein extracts from human heart, in which T cadherin was abundantly expressed, purchased from Clontech laboratory (Palo Alto, Calif.). Human lung proteins from a tumor-free region were also solubilized with lysis buffer. The protein was electrophoresed on SDS polyacrylamide gel electrophoresis (PAGE) gels and electroblotted to a nitrocellulose membrane. After blocking with fetal bovine serum, membranes were incubated with affinity-purified rabbit antibody to human T cadherin, goat antibody to SP-D, normal rabbit IgG, or normal goat IgG. Then, membranes were incubated with a second antibody labeled with horseradish peroxidase and developed with diaminobenzidine.

Detection of T cadherin and SP mRNA using reverse transcriptase polymerase chain reaction analysis

Total cellular RNA was prepared using RNA-zol B (Biotex Laboratory, Houston, Tex.). We used total RNA a as substrate for cDNA synthesis with reverse transcriptase polymerase chain reaction (RT-PCR) kit (Life Technologies, Gaithesburg, Md.). The cDNA synthesis and following PCR were performed according to the manufacturer's instructions. The primer sets used in this study are summarized in Table 1.

Expression vector, transfection, selection of A549 cells

In order to establish T-cadherin expressing cells, we used a human or murine T-cadherin expression vector constructed with pCI-neo vector (Promega, Madison, Wis.), as previously reported [23]. A549 cells were stably transfected using lipofection with vector alone or containing T-cadherin cDNA as described before [23]. T-cadherin-transfected cell lines were established after G418 selection (Sigma, St. Louis, Mo.).

Results

Reverse transcriptase polymerase chain reaction

The result of the RT-PCR analysis is shown in Fig. 1. Neither T cadherin, SP-A, nor SP-B mRNA was expressed in original and control plasmid-transfected A549 cells. In contrast, we observed SP-C and D mRNA expression in original and control-plasmid transfected A549 cells. We transfected A549 cells with the human T-cadherin expression vector and obtained more than ten colonies after G418 selection. Using RT-PCR, all of the G418-resistant A549 cell clones tested revealed the expression of T-cadherin mRNA. We could not detect SP-D mRNA expression in all of these human T-cadherin-transfected A549 cell clones as demonstrated in Fig. 1. We also transfected A549 cells with the murine T-cadherin expression vector. Again, using RT-PCR, no visible PCR product of SP-D was amplified with transfected A549 cells. Expression of the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene was monitored as the control in this examination.

Western immunoblotting

The Western immunoblotting results are demonstrated in Fig. 2 and Fig. 3. We detected two major T-cadherin

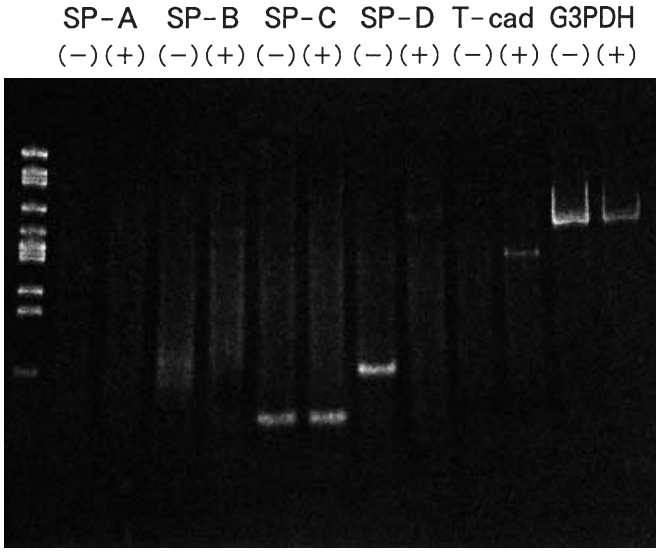


Fig. 1 Surfactant protein (SP)-A, B, C, and D mRNA expression in cultured A549 cells (human lung cancer cell line with alveolar type-II cell characteristics). Human T-cadherin expression vector-transfected (+) or control vector-transfected (–) A549 cells were examined using reverse transcriptase polymerase chain reaction analysis. Control vector-transfected A549 cells expressed SP-C and D mRNA but did not express SP-A or B or T-cadherin mRNA. After the transfection of human T cadherin, A549 cells gained T-cadherin mRNA expression and lost SP-D expression. G3PDH mRNA expression was monitored as a control

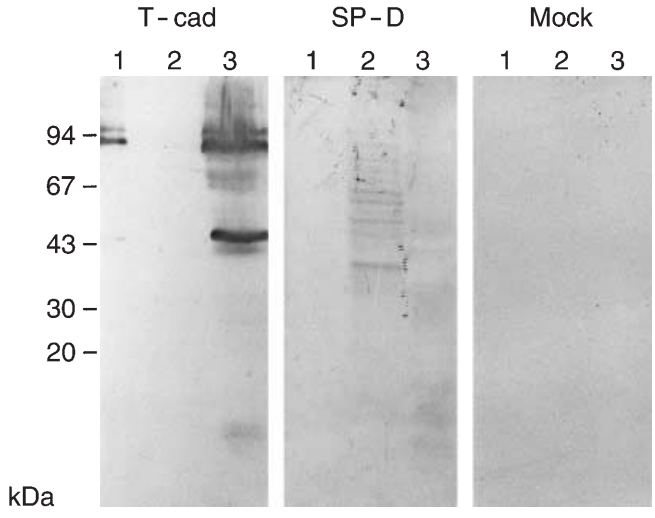
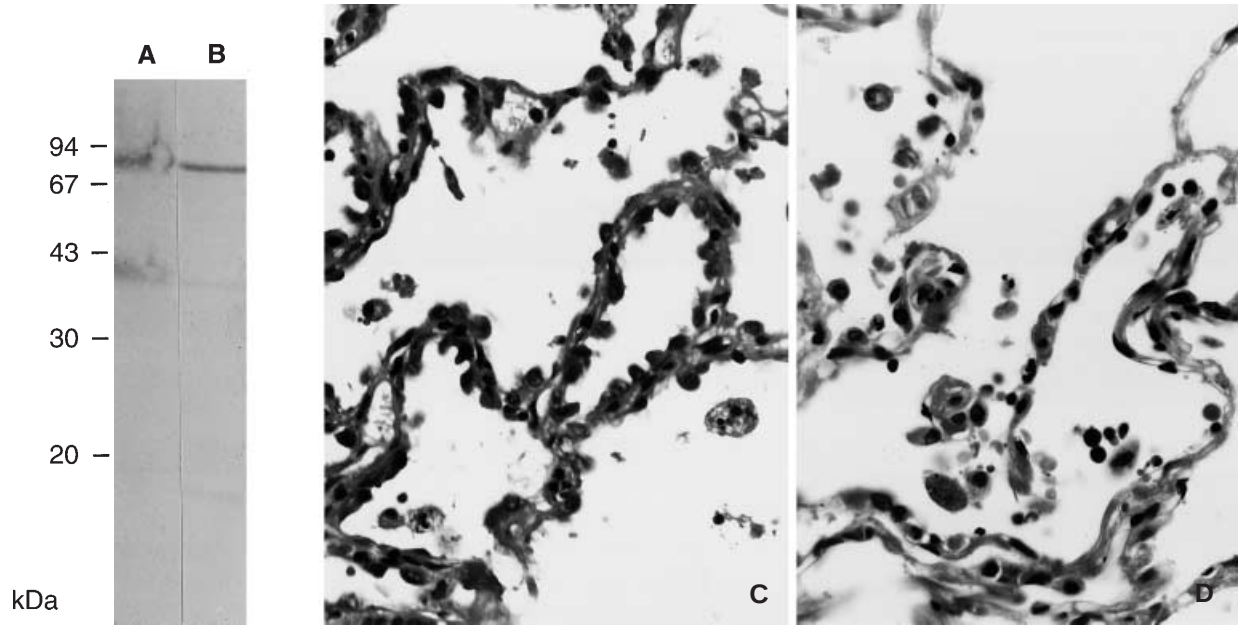


Fig. 2 Surfactant protein (SP)-D downregulation in human T-cadherin-transfected A549 (human lung cancer cell line with alveolar type-II cell characteristics) cells at the protein level. Human heart protein extracts (*lane 1*), control plasmid-transfected A549 cell lysates (*lane 2*), and T-cadherin expression vector-transfected A549 cell lysates (*lane 3*) were applied to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. Each membrane containing lanes 1–3 was incubated with anti-T-cadherin antibody or anti-SP-D antibody, or mock antibody. The mock antibody is a normal goat antibody. The blot is representative of four experiments. Normal rabbit antibody was also used as a mock antibody. No band was detected with the mock antibody in all four independent experiments



bands with approximate molecular weights of 80 kDa and 45 kDa in human T-cadherin-transfected A549 cells (Fig. 2). As expected, no significant bands were observed in control vector-transfected A549 cells with rabbit anti-T-cadherin antibody. A faint band with an approximate molecular weight of 40 kDa was observed in control vector-transfected A549 cells with goat anti-SP-D

Fig. 3 Western immunoblotting of human lung extracts with rabbit anti-T-cadherin antibody. The approximately 80-kDa and 45-kDa bands were observed in an alveolar type-II cell-rich specimen (**A**). In contrast, a single 80-kDa band was detected in another alveolar type-II cell-poor specimen (**B**). The microscopic appearance of each specimen was demonstrated (**C**, **D**). Original magnification ×400

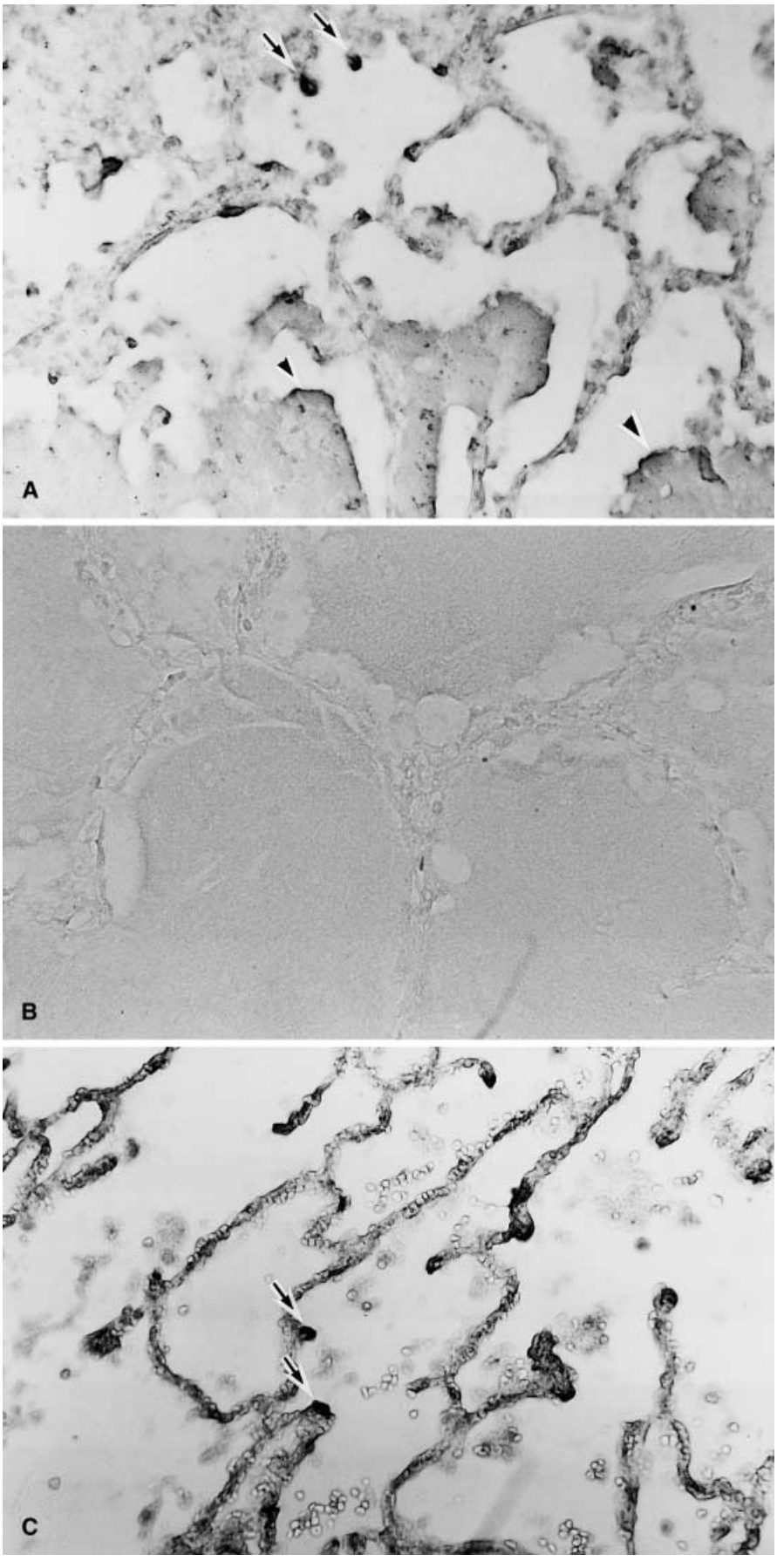


Fig. 4 Immunostaining for surfactant protein (SP)-D and T cadherin in lung specimens obtained from patients with secondary alveolar proteinosis (**A**, **B**) and lung cancer (**C**). We used a tumor-free region of the lung cancer specimens (**C**). In **A**, *arrow* and *arrowheads* indicate positive signals in alveolar type-II cells and the surface of an amorphous component, which filled the alveoli in a proteinosis specimen, respectively. Little or no positive signals were detected in alveolar cells from alveolar proteinosis using the anti-T-cadherin monoclonal antibody, MA-511 (**B**). In contrast, T cadherin was expressed in bronchioloalveolar type-II cells (*arrow*) from a tumor-free region of lung cancer specimens (**C**). Original magnification ×200 (**A**–**C**)

antibody. In contrast, no significant bands were detected in human T-cadherin-transfected A549 cells with anti-SP-D antibody. As expected, the T-cadherin band, with a molecular weight of 80 kDa, was observed using human heart extracts. Two T-cadherin bands, with an approximate molecular weight of 80 kDa and 45 kDa were also detected in a human lung extract (Fig. 3A). It is noteworthy that this lung specimen was from the tumor-free region of a lung-cancer patient. However, we observed a reactive proliferation of alveolar type-II cells, as demonstrated in Fig. 3C. In contrast, a single 80-kDa T-cadherin band was detected in another lung specimen (Fig. 3B) in which we could not see any significant proliferation of alveolar type-II cells (Fig. 3D).

Immunohistochemical staining

The results of the immunohistochemical staining are demonstrated in Fig. 4. SP-D was strongly expressed in alveolar type-II cells in specimens (Fig. 4A, *arrow*). The positive signals were detected on the surface of an amorphous component, which filled the alveoli (Fig. 4A, *arrowhead*). In contrast, little or no signal was detected in alveolar cells by the anti-T-cadherin monoclonal antibody, MA-511, in the identical proteinosis specimen (Fig. 4B). However, bronchioloalveolar type-II cells were immunostained with MA-511 in a control lung tissue (Fig. 4C, *arrow*). We also confirmed the results with rabbit anti-T-cadherin antibody. Reactivity was not detected with control antibodies.

Discussion

In the present study, we demonstrated that T cadherin downregulated SP-D expression in A549 cells. A549 cell is well characterized as having a feature of bronchioloalveolar type-II cells. A549 cells, cultured in our laboratory, expressed SP-C and SP-D as demonstrated in Fig. 1. Western immunoblotting also demonstrated that A549 cells expressed SP-D, as shown in Fig. 2. Pulmonary SP-D is a member of the collectin family and believed to be involved in antimicrobial defense [7]. SP-D also seems to have an immunomodulatory function, inhibiting T-lymphocyte proliferation and interleukin (IL)-2 production, specific IgE binding to allergens, and blocking allergen-induced histamine release from human basophils [1, 11]. Very recently, Madsen et al. showed that SP-D appeared to be generally present on mucosal surfaces and not restricted to a subset of cells in the lung [12]. They speculated that the function of SP-D can be the counterpart in the innate immune system to IgA in the adaptive immune system. Moreover, recent examination using SP-D-deficient mice suggested a critical role for SP-D in surfactant homeostasis [2]. Although these recent studies highlighted SP-D as an important molecule in both homeostasis and innate immunity, there are a few reports describing the mechanism of regulation of SP-D. Although SP-D has structural and functional similarities to SP-A, these two proteins are not coordinately regulated in response to lung infection. Rust et al. characterized the human SP-D promoter and emphasized the potential complexity of SP-D gene regulation [15]. Fisher et al. also speculated that SP-D expression is controlled by factors distinct from the level of SP-D in the alveolus by analysis of SP-D gene-targeted mice [6].

In the present study, we demonstrated that a unique member of the cadherin superfamily, T cadherin (CDH13, H cadherin), decreased the SP-D expression in A549 alveolar type-II cells in vitro. As demonstrated in Fig. 1, A549 cells transfected with the human T-cadherin expression vector decreased SP-D expression at the level of transcription. It is not surprising that murine T cadherin also decreased human SP-D expression, since the amino acid homology of T cadherin between human and mouse is over 95% [23]. Downregulation of the SP-D molecule by T-cadherin expression in A549 cells was also confirmed using Western immunoblotting (Fig. 2). Control vector-transfected A549 cells produced the SP-D molecule. We detected a band with an approximate molecular weight of 40 kDa in the control A549 cell extract using anti-SP-D antibody.

Although the dominant form of human SP-D is composed of monomeric subunits with a reduced molecular weight of 43 kDa, there is also a monomeric subunit with an apparent molecular mass of 50 kDa in some individuals [13]. We observed the other faint SP-D bands corresponding to a higher molecular mass in A549 cells. These bands may represent a multimeric form of SP-D [9]. Interestingly, two T-cadherin bands with approximate molecular weights of 45 kDa and 80 kDa were detected in T-cadherin-transfected A549 cells. We also detected the 45-kDa band in a human lung extract of a tumor-free region where alveolar type-II cells were reactively proliferating (Fig. 3A and Fig. 3C). In contrast, we detected a single 80-kDa T-cadherin band in another lung specimen, where a few alveolar type-II cells were observed. (Fig. 3B and D). Interestingly, we previously could not detect the 45-kDa protein with an identical antibody in human T-cadherin-transfected murine myeloma cells [23].

Taken together, the molecule with a molecular weight of 45 kDa may be a post-translational processing variant of T cadherin. This processing for a truncated form of T cadherin might be relatively restricted in A549 cells or bronchioloalveolar cells. Since A549 cells were from patients with bronchioloalveolar carcinoma and might not represent the nature of alveolar type-II cells, it is necessary to examine the relationship of T cadherin and SP-D in vitro or in vivo using the other alveolar type-II cells.

However, it is well known that alveolar type-II cells plated on plastic tissue culture containers spread rapidly and lose both their cuboidal morphology and expression of their genes for the SPs [18]. Moreover, the poor transfection efficiency of alveolar cells also make it difficult to examine the T-cadherin effect on SP-D production in vitro. It was a reason why we further examined the rela-

tionship of T cadherin and SP-D in alveolar proteinosis. Previously, Crouch et al. reported that human SP-D accumulated in the air spaces of patients with alveolar proteinosis [5]. As demonstrated in Fig. 4A, we observed strong expression of SP-D in alveolar type-II cells from four of four secondary alveolar proteinosis tested.

Interestingly, the surface of amorphous components, which filled the alveoli, were also stained with antibody to human SP-D. In contrast, T-cadherin expression was not observed in alveolar type-II cells in proteinosis (Fig. 4B). In parallel, we immunostained tumor-free area of lung tissues from patients with lung cancer. As shown in Fig. 4C, most alveolar type-II cells were strongly stained with anti-T-cadherin antibody. These data also support the competitive expression of T cadherin and the SP-D molecule in the lung.

Until now, we could not explain the precise mechanism of SP-D downregulation by T-cadherin expression. As described in the Introduction, T cadherin is a unique member of cadherin, because it lacks the cytoplasmic region, which is well-conserved in the other members of the cadherin family. This cytoplasmic region is necessary for the other cadherins to bind cytoskeletal protein, for example β-catenin[4]. However, highly conserved amino acids of T cadherin between various species indicates the fundamental biological property of T cadherin. It should be noted that morphological change was not observed in T-cadherin-transfected A549 cells used in this study (data not shown). Therefore, it is hard to speculate that previously reported homophilic adhesion mediated by T cadherin was directly linked to the downregulation of SP-D production. Combined together with the present data, it is possible to speculate that T cadherin had a unique role apart from cell adhesion, for example, regulated SP-D production in alveolar type-II cells.

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