Invasion Suppressor Role of E-Cadherin in Epithelial Cancer Cell Lines

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Background **: The generation of the invasiveness in transfromed cells represents an essential step of tumor progression. The primary cause of the scattering of the cells in invasive carcinoma is a loss of the integrity of the intercellular adherens junction often involving loss of a functional cell-cell adhesion molecule E-cadherin. Therefore, the perturbation of E-cadherin function causes diaggregation of tumor cells and may promote the invasion and metastases.**

Objectives **: The reduction in E-cadherin activity seems to correlate with the infiltrative ability of tumor cells. The purpose of this study was to compare the E-cadherin expression among different cell lines which were normal to undifferentiated and to check the virtual relationship between E-cadherin and invasiveness.**

Method : We used 5 cell lines, HaCaT, A431, C3, SiHa and HeLa cell. To check the expression patterns and amounts of E-cadherin in each cell line, immunofluorescence staining, Western blot anlysis and Northern blot analysis were done. An in vitro invasion assay using the collagen gel and MRC-5 fibroblast under the influence of HECD-1 antibody which block the E-cadherin function was done to measure the invasiveness of tumor cells. Collagenase activity in culture supernatants of each cell were analyzed by zymography.

Results **: Immunofluorescence staining revealed a homogenously well preserved pattern in HaCat, A431, C3 cells. SiHa cells showed patch distribution but HeLa cells did not express the E-cadherin. Western blot analysis and Northern blot results largely corresponded with the immunofluorescence results. The in vitro invasion assay revealed invasion into the collagen matrix of the HeLa cells. When HECD-1 antibody was added to the medium, other cells showed partially disrupted stratification. The collagenolytic activity at 72 kDa size was detected in the HeLa cell line only.**

Conclusion **: There is an inverse relationship between E-cadherin expression and tumor invasion. Therefore, through their regulation of cell adhesion and motility, cadherin plays a crucial role in the suppression of tumor invasion and metastasis.**

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Key Words : E-cadherin, Invasion

Cadherins are a family of cell adhesion molecules that are crucial for the cell-cell associations. Through their homophilic binding interactions, cadherins play a role as a morphogenetic regulator, they control cell polarization and take part in the formation of junctional complexes[1]. For the tumor cell invasion, tight cell associations must be disrupted and for the dissociation, the role of cadherins must be taken into account as they are crucial in connecting cells. There has been some evidence that the down-regulation of cadherin expression is associated with the invasiveness of tumors. This hypothesis is consistent with the observation

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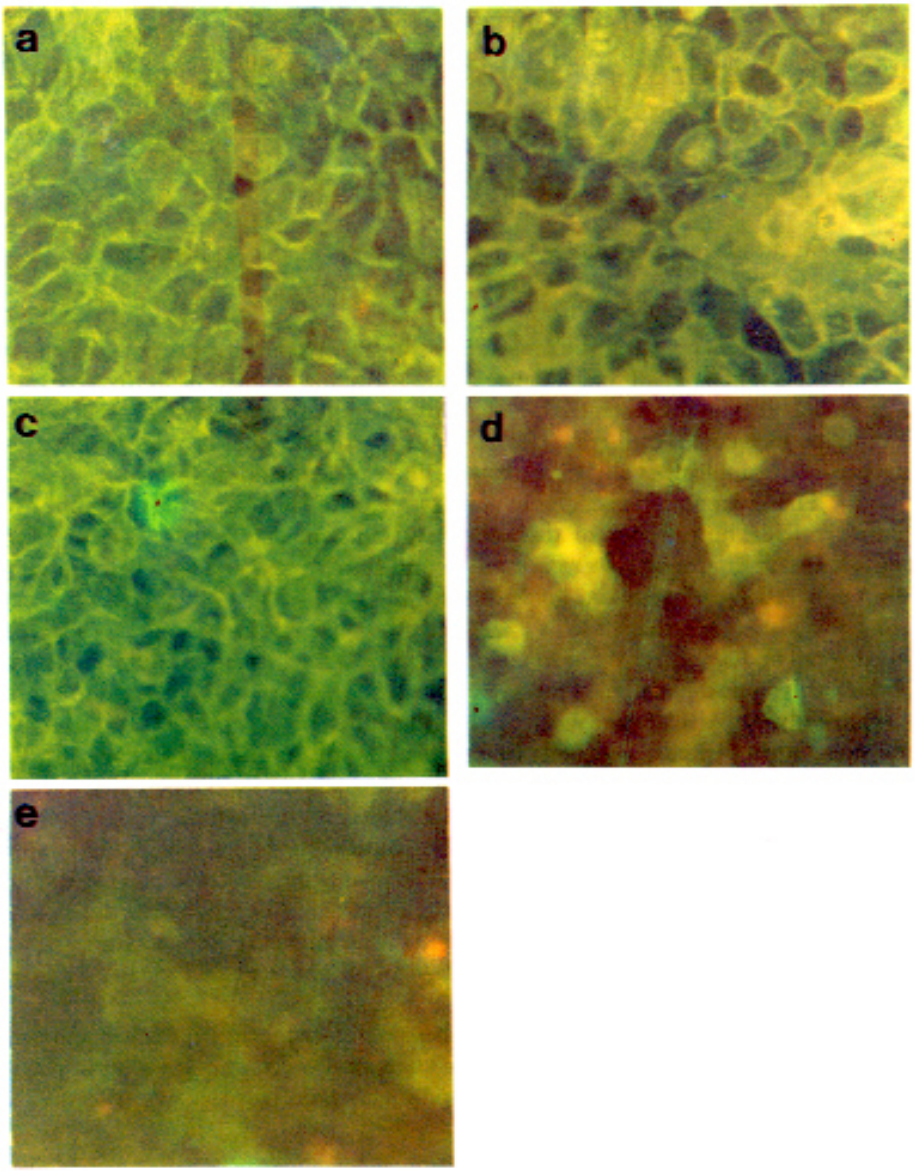

Fig. 1. Immunofluorescence staining of the cultured cells using HECD-1 antibody.)a ; HaCaT, b ; A431, c ; C3, d ; SiHa, e ; HeLa cells).

that malignant transformation of the normally noninvasive canine epithelial Madin-Darby canine epithelial kidney cell line with Harvey and Maloney sarcoma viruses was associated with the loss of expression of E-cadherin at the cell surface of the transformed cells[2]. Frixen et al.[3] showed that carcinoma cell lines with an epithelioid phenotype were noninvasive and expressed E-cadherin, whereas carcinoma cell lines with a fibroblastoid appearance were invasive and had lost E-cadherin expression. Cadherin expression in human cancers falls into 4 different types of tumor cells. Firstly, those that strongly and homogenously express cadherin. Secondly, those in which cadherin expression is reduced locally in the tumor resulting in a heterogenous distribution. Thirdly, those in which complete loss of cadherin and finally, those that express cadheirn but do not form aggregates. To

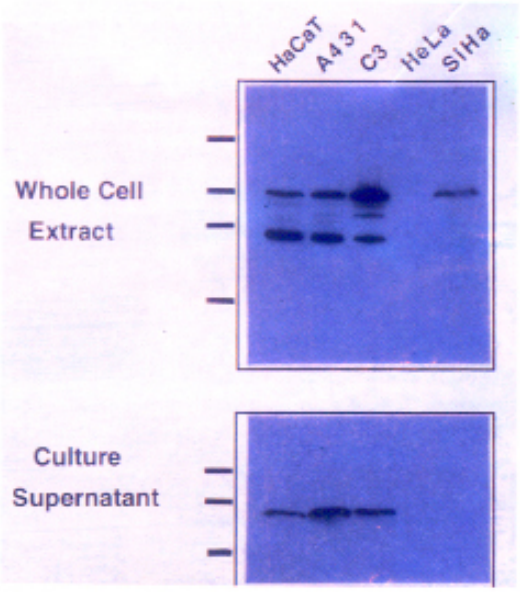

Fig. 2. Western bolt analysis of each cell extracts and cultured supernatants using HRCD-1 antibody.

address the last fourth type, it is possible that other factors might be involved in the cadherin dysfunciton[4]. Structural and functional analyses indicated that cytoplasmic anchorage through catenins is important in mediating adhesive interaction. Interestingly, *src* and *yes* proto-oncogenes and protein kinase C are colocalized with the cadherin-catenin complex in the zonula adherens, and these are involved in the tyrosine phosphorylation of α, ß-catenins. Also, epidermal growth factor induces tyrosine phosphorylation of ß, γ-catenins, resulting in release of the E-cadherin-catenin complex from the cytoskeleton[5].

The correlation between cadherin expression and metastsis incidence was reported for gastric, esophageal, breast, prostate and colorectal cancers and other squamous cell carcinoma of head and neck[6-13]. Although all-or-nothing phenomena were not found, there is a clear tendency that reduced types to be more infiltrative than preserved types and showed higher frequencies of lymph node or regional and distant metastasis.

The purpose of this study was to compare the E-cadherin expressions among different cell lines which were normal to undifferentiated and to elucidate the virtual relationship between E-cadherin

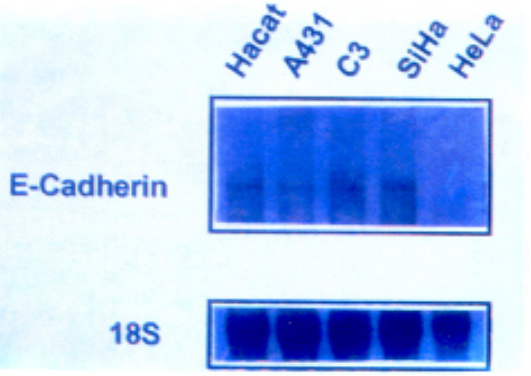

Fig. 3. Northern blot analysis of total RNA extracts of cells probed with HC6-1 E-cadherin cDNA. 18S RNA amount was used with internal control.

and invsiveness.

MATERIALS AND METHODS

Cell lines

Five different cell lines were used. HaCaT cells are immortalized normal human keratinocytes(gift from Dr. Nobert Fusenig)[14], A431 cells are well differentiated vulvar epidermoid carcinoma cells(ATCC, #CLL-1555, Bethesda, MD), C3 cells are cervical adenocarcinoma cell lines, the HeLa cell is a human epidermoid cervical carcinoma cell(ATCC, #CCL-2, Bethesda, MD), SiHa cells are human cervical squamous cell carcinoma cell lines(ATCC, #HTB-35, Bethesda, MD). HaCaT cells and HECD-1 hybridoma cells were cultured in 1:1 mixture of Dulbecco's modified Eagles Medium(DMEM) and Ham's F-12 medium plus 10% fetal calf serum and other cell lines were cultured in DMEM.

Immunofluorescence

Cells were cultured in cover glasses and fixed with 3% paraformaldehyde for 5 minutes and permeated with ice cold 0.2% Triton-X100 in PBS. After washing with PBS three times, they were incubated with blocking solution(0.2% BSA in PBS) for 10 minutes. Then cells were incubated or 1 hour at 37℃ with culture supernatant of HECD-1, which is a mouse hybridoma clone producing monoclonal antibodies to human E-cadherin(generously provided by Dr. Mashtoshi Takeichi). After washing with PBS three times, fluorescein conjugated

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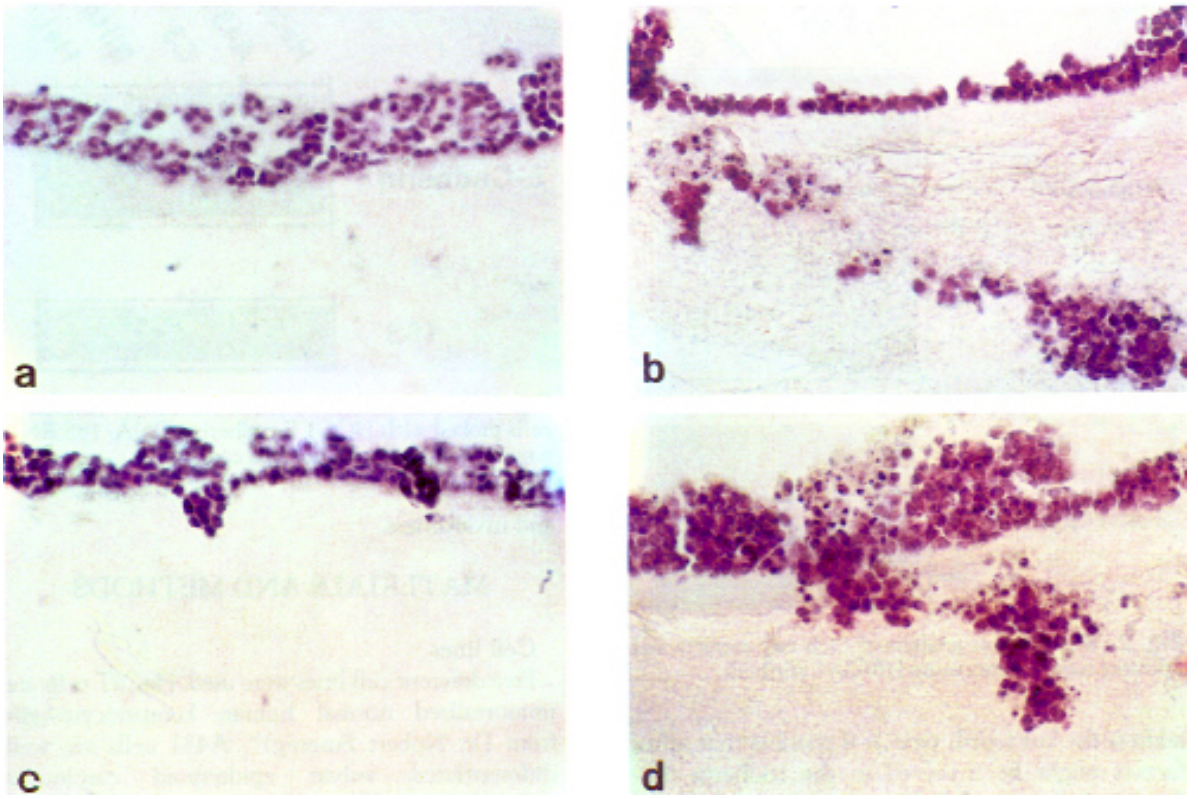

Fig. 4. Invasion assay of HaCaT and HeLa cells (a and b; HaCaT cells and HeLa cells in the absence of HECD-1 Ab, c and d; 3 days and 14 days after addition of HECD-1 Ab(10μg/ml) in HaCaT cell raft cultures).

anti-mouse IgG(Cappel, Durham, NC, USA) was applied to the cells for 30 minutes at 37℃.

Western blot Analysis

Each cell lysate was prepared in extraction buffer containing 1.5% SDS, 62.5mM Tris-HCl(pH 7.0), 5% 2-mercaptoethanol, 2mM PMSF, 2mM $CaCl_2$, 10μg/ml pepstatin A, 10μg/ml antipain, 10μg/ml chymostatin. 10μg of the proteins were diluted in a sample buffer(20% glycerol, 10% ß-mercaptoethanol, 0.5% bromophenol blue, 4.6% SDS, 0.125M Tris.HCl, pH 6.8), then boiled for 5 minutes and separated on 8% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membrane(Bio-Rad, Hercules, CA, USA) and blocked with 5% skim milk in TBS buffer(150mM NaCl, 50 mM Tris-HCl, pH 7.6) for 1 hour and incubated with HECD-1 culture supernatant for 1 hour at room temperature. After washing with TBS buffer, it was incubated with peroxidase conjugated anti-mouse IgG(1:3000) for 1 hour. Visualization was perfromed using chemiluminescence(Amersham, Buckinghamshire, UK).

Northern blot Analysis

Total RNAs were extracted from cells using RNAzol™ B solution(Biotecx, Houston, TX, USA) according to the manufacturer's instruction. Briefly, cells grown in a monolayer in the culture dish were solubilized in RNAzol B solution. After 1/10 volume of chloroform was added to the lysates, samples were vigorously shaken, and centrifuged at 12,000g(4℃) for 15 minutes. Then upper aqueous phases were collected and precipitated with ethanol. 10μg of each RNA was electrophoresed in 1% agarose gel, then transferred to Hybond-N nylon membrane(Amersham) and probed with 386 base pair sized human E-cadherin cDNA, HC6-1, which was kindly provided by Dr. Walter Birchmeier[3]. An 18s rRNA probe was used as a control for the total amout of

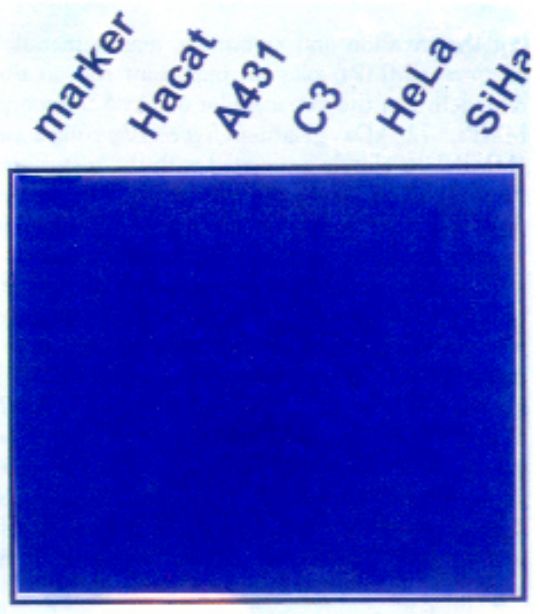

Fig. 5. Gelatin zymogram of conditioned medium from each cell lines. 72-kDa sized gelatinolytic acitvity is seen only in the HeLa cell lane.

RNA.

In vitro Invasion Assay

In a 24 well culture plate, 3×10^5 cells in 200μl were seeded on the collagen gel and Cell Matrix Type I-A(Nitta Gelatin, Yao-shi Osaka, Japan) in a Millicell culture plate insert(Milipore, Bedford, MA, USA) was also used containing MRC-5 human lung fibroblasts(provided from Japanese Cancer Research Resources Bank) which are known to secrete scatter factors to promote the invasion. Then the DMEM/F12 media were changed every other day. After 5 days of incubation, HECD-1 antibody(10μg/ml) was added to the media to block the E-cadherin function. After 14 days of incubation, the gels were fixed with 10% formaldehyde and stained with hematoxylin and eosin.

Assay for collagenase type IV

Collagenase activity in the 24-hour conditioned serum-free culture supernatant of each cell was assayed as described before[13]. Briefly, conditioned media were subjected to SDS-PAGE in a gel containing 10% acrylamide and 0.1%(wt/vol) gelatin. In one experiment, conditioned medium of HeLa cells were previously treated with 50μg/ml trypsin for 1 hour at 37℃ to activate the latent enzymes. Following removal of SDS, the gel was incubated in the developing buffer(10mM Tris base, 40mM Tris-HCl, 200mM NaCl, 0.02% Brij 35, 5mM $CaCl_2$) for 3 days at 37℃. In one experiment the developing buffer was supplemented with 10 mM EDTA. Then it was stained with Coomasie blue and destained.

RESULTS

Expression of E-cadherin in cell lines

The immunofluorescence staining result revealed that HaCaT cells, A431, C3 showed a homogenous well preserved pattern and E-cadherin was especially concentrated along the cell borders. In contrast, SiHa cells showed patch distribution of E-cadherin. However, HeLa cells did not express the E-cadherin(Fig. 1). Western blotting analysis demonstrated that the total amounts of E-cadherin in these cells largely corresponded with the immunofluorescence data. HaCaT, A431, C3 cells showed 120 kDa band and 80 kDa trypsin-resistant fragments. HeLa cells, however, did not show any band. C3 cells expressed higher amounts of E-cadherin compared to HaCaT and A431 cells(Fig. 2). Northern blot analysis revealed similar results of E-cadherin amounts at mRNA levels(Fig. 3).

In vitro invasiveness of cells

HaCaT, A431, C3 and SiHa cells stratified on the collgen gels and did not invade the gel, whereas HeLa cells invaded the collagen matrix. Three days after HECD-1 antibody was added into the medium, which is the functional blocking antibody to E-cadherin, cells showed partially disrupted stratification and began to invade the collagen matrix(Fig. 4). However, HeLa cells showed the same invasiveness pattern as those without HECD-1 antibody.

Collagenase activity in HeLa cells

The capacity to secrete collagenolytic enzymes was analyzed by a zymogram of the culture supernatant of each cell. The gelatinolytic activity at 72 kDa size was detected in HeLa cell lines only. Gelatin degrading enzyme were visualized as a clear band resulting from proteolysis of gelatin(Fig. 5). This band was cleaved with 50μg/ml trypsin activa-

tion to a latent and an activated faster band and was abolished by 10 mM EDTA, establishing them as metalloprotease.

DISCUSSION

The actual importance of normal cadherin expression in regulating the invasive behavior of epithelial cells was first suggested by Behrens et al[2]. They found that when the intercellular adhesion of a canine epithelial cell line was disrupted by the addition of anti E-cadherin antibody, which blocks the function of E-cadherin, the normally non-invasive MDCK cells acquired the ability to invade the collagen gels and embryonal heart tissue. We also found the similar effect of anti E-cadherin antibody in blocking the invasion suppressive function of E-cadherin in non-invasive HaCaT, A431, C3, SiHa cells. A direct role of cadherins was demonstrated when the invasiveness of these cells could be prevented by transfection with E-cadherin cDNA. Vleminckx et al.[15] transfected E-cadherin cDNA into highly invasive tumor lines and caused a decrease in an in vitro invasion assay and production of in vivo tumors that were more differentiated than those produced by non-transfected cells. Good experimental evidence exists to support the hypothesis that the loss of cadherin expression or function is associated with increased invasiveness in transformed epithelial cells. However, these observations have not been consistently confirmed in immunohistochemical analysis of human tumors. It is possible that the other host factors could play important roles in the heterogenous nature of cadherin expression.

One of the important aspects of the function of cadherins is that they form a complex with the cytoskeleton and the tyrosine kinases of the *src* family are localized to this complex. This raises the possibility that cadherin-mediated cell cell junctions might be used for intercellular signalling. It is suggested that phosphorylation and dephosphrylation of catenins, linker proteins between E-cadherin and microfilament, regulate the ratio of free and actin-bound cadherin-catenin complexes, thereby regulating the adhesive forces of cadherin[13, 16,17]. There was evidence that reduction of α-catenin expression in spite of normal expression of E-cadherin was significantly associated with tumor dedifferentiation, infiltrative growth and lymph node metastasis[12,18]. For the invasion and metastasis, matrix metalloprotinases(MMPs) play an important role in the remodeling of the extracellular matrix[19, 20]. Among MMPs, 72 kDa gelatinase/type IV collgenase (MMP-2) is closely associated with the metastatic phenotype because this is a neutral MMP capable of degrading basement membrane(type IV) collagen. It was observed that type IV collagenase expression is increased in colon adenocarcinoma and ovarian tumors[21,22]. With our results of gelatin zymogram with 24 hour conditioned serum-free culture supernant, we could see the gelatinolytic activity at 72 kDa size only in the HeLa cell lines. To confirm that this band is metalloprotease, we treated the supernatant with 10mM EDTA and different concentrations of trypsin. With EDTA treatment of the supernatant, the band was abolished and with 50 μg/ml trypsin activation, the band was cleaved to a latent and activated faster band.

According to our results, among these 5 cell lines, HaCaT, A431, C3, SiHa cells, which showed homogenous or heterogenous types of E-cadherin expression, they did not show the invasion activity in the collagen gel marix. However, HeLa cells which had no expression of E-cadherin revealed an invasive character. Although there are many other factors involved in tumor invasion, through their regulation of cell adhesion and moility, cadherins play a crucial role in the suppression of tumor invasion and metastasis.

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