Inverse relationship between E-cadherin and p27^{Kip1} expression in renal cell carcinoma

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Abstract. The cell-cell adhesion system plays a pivotal role in the maintenance of tissue structure and cell-cell communication. E-cadherin is a major adhesion protein of the epithelial cells, and E-cadherin expression may be involved in the regulation of cell proliferation or differentiation. To address the relationship between cell-cell adhesion and cell proliferation, we focused on the alteration of p27^{Kip1} (p27), a cyclin-dependent kinase inhibitor, by E-cadherin-mediated adhesion. In an immunohistochemical study of 76 cases of renal cell carcinoma (RCC), the p27-labeling index (LI) was 67% in E-cadherin-reduced RCC, but only 28% in E-cadherin-preserved RCC. E-cadherin-expressing cells rarely expressed p27 in various cancers including those of the breast, colon, liver and prostate. In a subconfluent monolayer culture, the E-cadherin levels were increased steadily in the E-cadherin positive RCC cell line ACHN, whereas the p27 levels were decreased. Subsequent exposure of ACHN cells to the E-cadherin-specific function-blocking antibody reduced the growth associated with the increase in p27 and the decrease in phosphorylated epidermal growth factor receptor (EGFR). In the E-cadherin negative RCC cell line Caki-1, these effects were not observed. These results suggest that E-cadherin-mediated adhesion may be involved in the contact stimulation for cell proliferation in part through the downregulation of p27 and the activation of EGFR in human cancers.

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Introduction

The adherence junction at the cell membrane plays a pivotal role in the maintenance of cell phenotype and tissue formation during development and differentiation. E-cadherin is a transmembrane glycoprotein that mediates calcium-dependent cell adhesion through homophilic interaction with the E-cadherin expressed on apposite epithelial cells (1). The intracellular domain of E-cadherin binds with the actin cytoskeleton via a protein complex containing α-, ß-, γ-catenins and p120CAS. Thus, E-cadherin maintains tissue stability through cell rearrangement, cell migration, and tissue formation in a cell contact-dependent manner. It has also been demonstrated that the E-cadherin-mediated adhesion system is subject to outside-in signaling through mitogen-activated protein kinase (MAPK) pathways (2,3). Accordingly, E-cadherin would seem to regulate cell proliferation, differentiation, and survival (4-6); however, little is known about its precise molecular mechanism.

Cell-cycle progression is controlled by a series of kinase complexes composed of cyclins and cyclin-dependent kinases (CDKs). These regulatory mechanisms are involved in cyclin levels, the state of phosphorylation of CDKs, and the actions of CDK inhibitors. p27^{Kip1} (p27) is a universal CDK inhibitor that directly binds with CDK2/cyclin E complex, and consequently G1 arrest occurs (7). It is well known that p27 accumulates in serum-starved and density-arrested cells and plays a pivotal role in the control of cell proliferation. Indeed, a large number of studies have validated the utility of p27 as a prognostic or diagnostic marker in a variety of human malignancies (8,9).

We report for the first time the significant inverse relationship between E-cadherin and p27 expression in various human cancers including renal cell carcinoma (RCC). Furthermore, the loss of E-cadherin function resulted in the retardation of cell proliferation associated with the increased p27 protein levels and the inhibition of EGFR. These findings suggest that E-cadherin may be able to function as a downregulator of p27 *in vivo* and may thereby control cell proliferation.

Materials and methods

Cell culture. The human Caki-1 and ACHN renal cell carcinoma cell lines were all obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer belonging to Tohoku University. Cells were maintained in RPMI-1640 medium supplemented with 10% FCS and antibiotics. The culture medium was changed every 2 days and the number of cells at various times was scored using a hemocytometer. Dead cells, as determined by 0.3% trypan blue staining, were left out of the count. Cells were seeded in triplicate in all experiments. When confluent, cells were trypsinized and seeded onto 60-mm dishes with defined media for use as antibodies in functional studies. Two days after plating, the cells reached the logarithmic phase and were then cultured with 1 μg/ml of SHE78-7 E-cadherin-neutralizing antibody (Takara, Shiga, Japan) or IgG2a isotype-matched control mouse IgG (Dako, Glostrup, Denmark). SHE78-7 binds with the extracellular domain of E-cadherin and disturbs the function of cell adhesion. In addition, for the purpose of blocking E-cadherin function which is dependent upon calcium concentration, cells were also cultured in medium containing 0.2 mM EDTA, as previously described (10). Cells were collected and assayed 48 h after treatment with or without SHE78-7 or EDTA.

RT-PCR. Using RT-PCR, human E-cadherin mRNA was assessed in all the cell lines. Total RNA was isolated using TRIzol reagent (Gibco BRL, Rockville, MD) according to the manufacturer's protocol. Reverse transcription was performed with 5 μg of total RNA in a total volume of 20 μl containing Superscript II reverse transcriptase (Gibco BRL). The PCR reagents, including 2.5 U of Taq DNA polymerase (Gibco BRL), were added to form a final volume of 50 μl. The PCR was performed in a Gene Amp PCR System 9600 (PerkinElmer, Foster City, CA). The 35-cycle amplification profile consisted of denaturation at 94˚C for 1 min, annealing at 56˚C for 1 min, and extension at 72˚C for 1 min. Primers used for E-cadherin were as follows; forward 5'-GACGCG GACGATGATGTGAAC-3' and reverse 5'-TTGTACGTG GTGGGATTGAAG-3'. Human ß-actin primers were utilized as positive internal controls. Negative controls without RNA and without reverse transcriptase were also utilized. The PCR product was electrophoresed in 2.0% agarose gel and visualized with ethidium bromide.

Immunoblotting. All the cell lines were cultured with medium containing SHE78-7, control IgG and EDTA. Forty-eight hours after treatment, cell lysates were prepared by homogenization in Triton X-100 buffer (50 μM HEPES, 150 μM NaCl, 1% Triton X-100, and 10% glycerol containing 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM sodium vanadate) and proteinase inhibitors. The protein concentration of each lysate was determined using a protein assay reagent kit (Dojindo Laboratories, Kumamoto, Japan). The total cell lysate was applied on 7.5% SDS-PAGE. After electrophoresis, the proteins were transferred electrophoretically from the gel to Immobilon membranes (Millipore, Bedford, MA). The membranes were then blocked for 30 min in blocking buffer (5% low-fat dried milk in Tris-buffered saline) and probed with the primary antibodies. After being washed, the protein content was made visible by horseradish peroxidase-conjugated secondary antibodies followed by enhanced chemiluminescence (ECL; Amersham). The primary antibodies used were raised against human E-cadherin, ß-catenin, $p27^{Kip1}$ (all from Transduction Laboratories), $p21^{Cip1/Waf1}$ and cyclin E (both from Calbiochem, Cambridge, MA), and phosphorylated EGFR (Upstate Biotechnology, Inc.).

Immunohistochemistry. For immunohistochemistry, we used formalin-fixed and paraffin-embedded materials obtained from 76 patients with RCC. These cases consisted of two pathological types of RCC, 67 cases of clear cell type (conventional) RCC, which had been investigated in an earlier study (11), and 9 cases of chromophobe cell type RCC. In order to represent the data of other cancers, 5 cases of colon, 7 cases of breast, 6 cases of liver (hepatocellular carcinoma) and 5 cases of prostate cancer were randomly selected.

Sections (5-μm thick) of paraffin-embedded tissues were mounted on poly-L-lysine-coated slides, dewaxed, rehydrated, and incubated for 30 min with 0.3% hydrogen peroxide in order to quench the endogenous peroxidase activity. All the slides were processed to unmask the antigens by being microwaved in 10 mM sodium citrate buffer (pH 6.5) for 5 min, three times, and by subsequent detergent treatment using PBS(-) containing 0.05% Tween-20 for 30 min. The slides were then treated with 10% normal rabbit serum (Nichirei, Tokyo, Japan) for 30 min, to reduce background staining, after which they were treated with the primary monoclonal antibodies against E-cadherin and p27 (Novocastra, Newcastle upon Tyne, UK) at 4˚C overnight. The slides were washed in PBS(-) twice and developed using the labeled streptavidin biotinylated peroxidase method (Nichirei) according to the manufacturer's instructions. 3,3'-diaminobenzidine tetrahydro-chloride (DAB) was used as the chromogen and hematoxylin as the counterstain.

Following a method described previously (12), we evaluated only membranous E-cadherin expression such as (-), (-+), (+) and we then divided the findings into two groups, consisting of a reduced group and a preserved group. In addition, nuclear p27 was evaluated using a labeling index (LI), which shows the percentage of positive tumor cells, as previously described (11).

We also performed double immunostaining in clear cell RCC, colon, breast, and hepatocellular carcinomas and in normal prostatic tissue using a peroxydase for E-cadherin and an alkaliphosphatase for p27 as the substrate.

Statistical analysis. Statistical comparisons were performed using the Mann-Whitney U test; $P<0.05$ was considered significant.

Results

Inverse correlation between E-cadherin and p27 expression in human malignant tissues. In the normal renal cortex, E-cadherin was only localized in the membrane of the distal tubules, whereas strong positive nuclear staining of p27 was

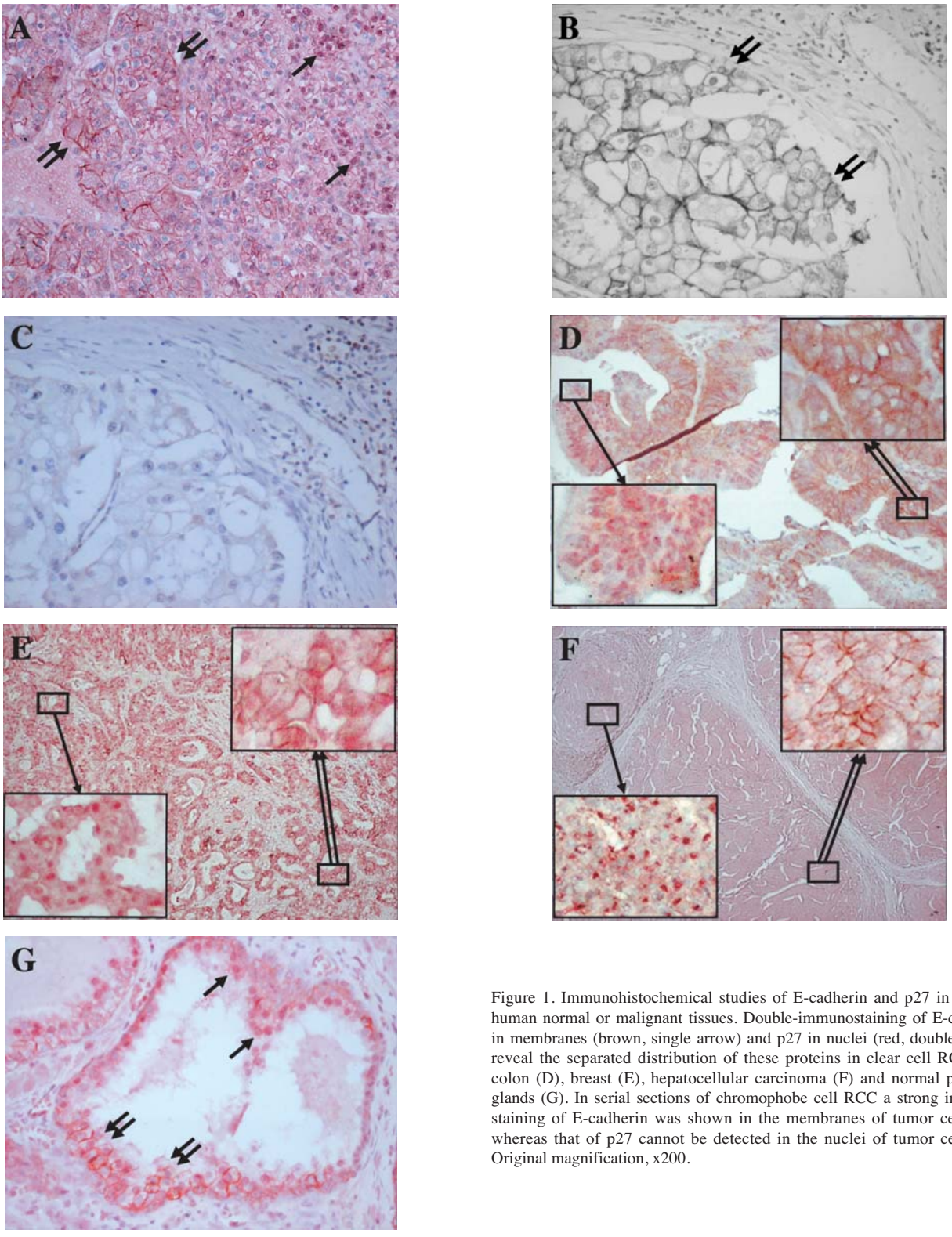

Figure 1. Immunohistochemical studies of E-cadherin and p27 in various human normal or malignant tissues. Double-immunostaining of E-cadherin in membranes (brown, single arrow) and p27 in nuclei (red, double arrow) reveal the separated distribution of these proteins in clear cell RCC (A), colon (D), breast (E), hepatocellular carcinoma (F) and normal prostatic glands (G). In serial sections of chromophobe cell RCC a strong immunostaining of E-cadherin was shown in the membranes of tumor cells (B), whereas that of p27 cannot be detected in the nuclei of tumor cells (C). Original magnification, x200.

localized in the distal and proximal tubules, glomerular epithelial cells and lymphocytes (data not shown).

In the clear cell type RCC, the tumor cells expressing E-cadherin tended to be distributed within the center of the tumor, whereas p27 tended to be located at the peripheral area of the tumor. Double-stained immunohistochemistry with E-cadherin and p27 antibodies showed a clearly separated distribution of these proteins in the tumor cells, and that E-cadherin-expressing cells rarely expressed p27 in their nuclei, and vice versa (Fig. 1A). Furthermore, it is worth noting that p27 LI was near zero in chromophobe cell type RCC, which was well characterized by a strong E-cadherin expression, in all nine cases (Fig. 1B and C).

The relationship between the levels of p27 and E-cadherin was analyzed by the Mann-Whitney U test in 67 cases of clear cell RCC. In clear cell RCC, 77.6% of the cases

Table I. The relationship between E-cadherin and p27 in various cancers.

	Colon cancer		Breast cancer		HCC		Prostate cancer	
E-cadherin (n)	Red. (1)	Pre. (4)	Red. (3)	Pre. (4)	Red. (2)	Pre. (4)	Red. (1)	Pre. (4)
p27 LI (%)	5.1	0.2	24.5	13.1	0.1	0.1	17.1	4.6

Red., E-cadherin-reduced group; Pre., E-cadherin-preserved group; HCC, hepatocellular carcinoma.

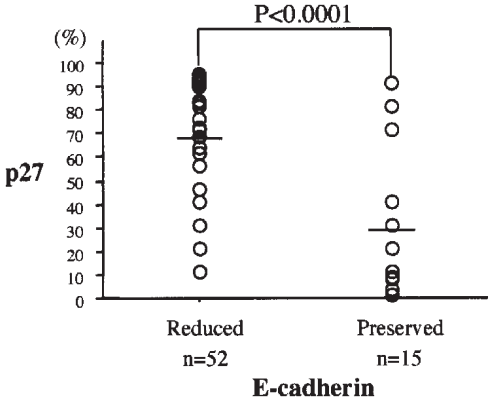

Figure 2. Comparison of p27 LI and E-cadherin expression in clear cell RCC. Mean value of p27 LI in the E-cadherin-reduced group was 67%, in contrast to 28% in the E-cadherin-preserved group. A statistical difference between them was obtained by Mann-Whitney U test (P<0.0001).

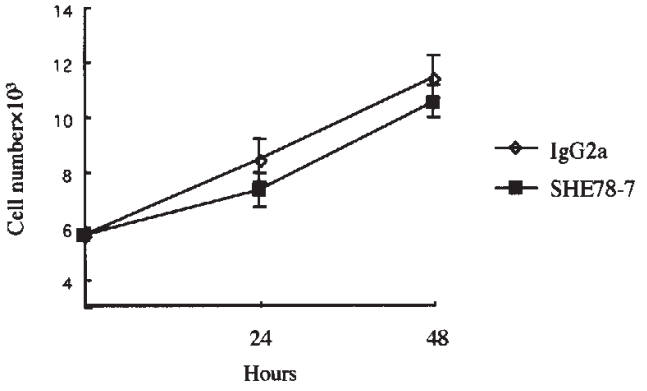

Figure 4. A proliferation assay of ACHN cells with control IgG2a or SHE78-7 antibody. Cells were harvested at the logarithmic phase and then plated them onto 60-mm dishes with control IgG2a or SHE78-7 antibody. Cell number was counted with 0.3% trypan blue staining and dead cells were excluded. The experiment was performed triplicate.

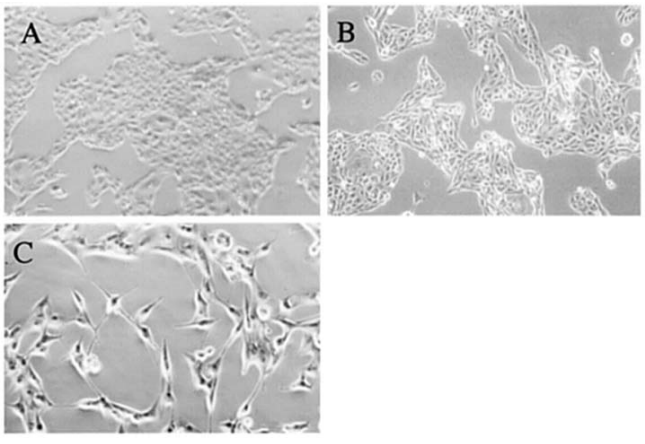

Figure 3. Phase-contrast micrographs of ACHN (A and B) and Caki-1 (C) cell lines in the presence of control (A and C) or SHE78-7 (B) antibody. E-cadherin-expressing cells, ACHN, show epithelial-like phenotype, whereas E-cadherin-non-expressing cells, Caki-1, show fibroblastic phenotype. SHE78-7 antibody induced the distinct cell-cell borders in ACHN cells 48 h after the treatment (B).

belonged to the E-cadherin-reduced group and the mean value of the p27 LI was 67%. In contrast, 22.4% of the cases belonged to the E-cadherin-preserved group, and the mean value of the p27 LI was 28%. A statistical difference between them was obtained at P<0.0001 (Fig. 2). In a small number of other cancers, a similar inverse relationship between E-cadherin and p27 was noted in colon, breast, hepatocellular and prostate cancers (Fig. 1D-G and Table I).

E-cadherin neutralizing antibody disrupts intercellular contacts. ACHN cells exhibited an epithelioid phenotype in a monolayer culture (Fig. 3A), whereas Caki-1 demonstrated a fibroblastoid phenotype (Fig. 3C). To demonstrate the E-cadherin expression levels of these cell lines, we performed RT-PCR and Western blot analysis for E-cadherin. As a result, ACHN cells expressed E-cadherin in both mRNA and protein levels, but Caki-1 expressed neither the mRNA (data not shown) nor its E-cadherin protein.

Cells growing in the control IgG-containing medium were tightly associated with each other, and their cell-cell borders were less distinct (Fig. 3A). Forty-eight hours after adding SHE78-8 or EDTA in the logarithmic phase, although the cells continued to maintain cell-cell adhesion, the cell-cell borders of E-cadherin-positive cells became more visible (Fig. 3B). This morphological change was not observed in Caki-1 cells (data not shown).

E-cadherin neutralizing antibody inhibits cell growth. We tested whether SHE78-7 was able to affect the proliferation of ACHN cells in a subconfluent monolayer culture. The SHE78-7 antibody resulted in the suppression of cell growth in ACHN cells, compared to the control IgG (Fig. 4). This experiment was performed three times with similar results. The results therefore suggest that E-cadherin is required for proliferation.

Differential expression of E-cadherin, p27, and cyclin E in vitro. To determine the kinetics of change in E-cadherin and p27 during cell growth in culture, the levels of these

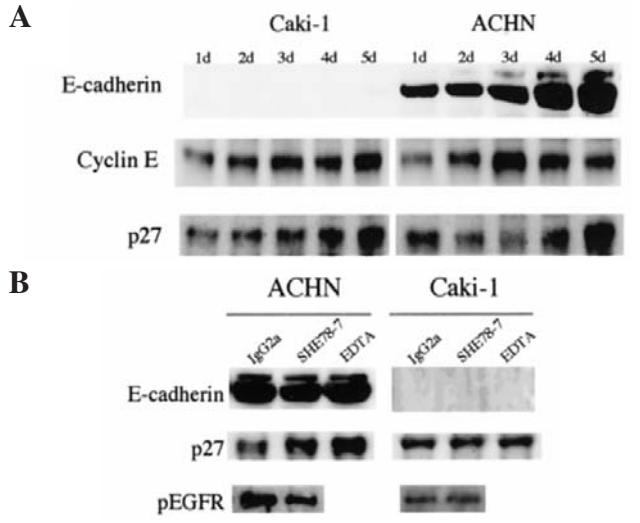

Figure 5. (A) Time course demonstrating changes in E-cadherin, cyclin E, and p27 protein levels during ACHN and Caki-1 cell growth over 5 days. Both cyclin E and p27 levels gradually increased during Caki-1 cell growth. However, ACHN cells showed reciprocal protein levels between cyclin E and p27, and a gradual increase in E-cadherin during cell growth. (B) Changes in p27 and phosphorylated EGFR protein levels of ACHN and Caki-1 cells in response to IgG2a, SHE78-7, and EDTA. Increased p27 and decreased phosphorylated EGFR levels were displayed after treatment of SHE78-7 or EDTA in ACHN cells. In contrast, there were no changes in Caki-1 cells.

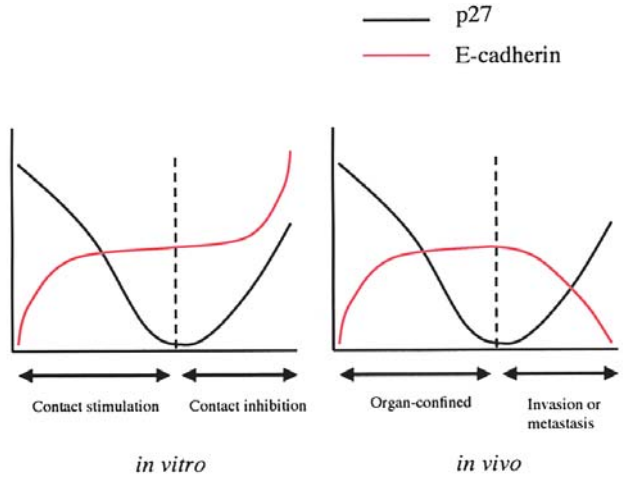

Figure 6. A schema of E-cadherin and p27 levels *in vitro* and *in vivo*. E-cadherin expression *in vitro* was markedly accumulated in the plateau phase. In contrast, E-cadherin expression was reduced at the advanced stage *in vivo*. Inverse relationship between E-cadherin and p27 *in vivo* estimated in both organ-confined and advanced stage.

proteins were assayed at various time points in the logarithmic phase. Two days after plating at $3x10^5$ cells, the cells were allowed to reach the logarithmic phase, and thereafter cells reached confluence within 7 days. Although E-cadherin levels were increased in a time-dependent manner in ACHN cells, p27 levels were decreased during the first 3 days, and were increased progressively thereafter (Fig. 5A). This rapid increase in p27 at the late plateau phase may account for the contact inhibition. In comparison with p27 levels, cyclin E levels were increased within 3 days in ACHN cells, but decreased thereafter, corresponding with the downregulation of the cyclin E-CDK2 complex by p27. In contrast to ACHN, p27 levels were not changed in Caki-1.

E-cadherin neutralizing antibody induces the increase of p27 and EGFR inhibition. To determine whether E-cadherin affects p27 expression, we compared p27 protein levels in ACHN cells treated with control IgG, SHE78-7, and EDTA. When ACHN cells were incubated with SHE78-7 or EDTA, p27 levels were increased and phosphorylated EGFR was decreased, compared to cells incubated with control IgG (Fig. 5B). This effect of SHE78-7 or EDTA was dose-dependent (data not shown). In addition, neither p21 nor ß-catenin levels were changed under the same treatment (data not shown).

These results suggest that the downregulation of p27 by E-cadherin-mediated cell-cell adhesion suppresses p27 expression, in part, through the decrease of phosphorylation of EGFR.

Discussion

E-cadherin, whose function is critical for cell-cell adhesion and developmental morphogenesis, has also been implicated in tumorigenesis, specifically as an invasion- or progression-suppressor protein (13). In recent years, however, there is growing evidence that E-cadherin is involved not only in cell-cell contact, but also in the regulation of cell proliferation, survival, and differentiation. It has been reported that E-cadherin inhibits cell proliferation through the upregulation of p27 in breast, colon and lung cancer cell lines (14). In confirmation of the function of p27 as a suppressor of cell proliferation, several clinical studies have correlated low p27 expression with aggressive cell proliferation and with poor prognosis in various malignancies (8,9). p27 protein is mainly regulated at the post-translational level via ubiquitination or specific proteolysis (15-17). However, multiple and distinct mechanisms of p27 regulation have also been suggested (9).

In this study, we identified possible p27 regulation by the E-cadherin-mediated adhesion system *in vitro*, and *in vivo*.

It may seem unlikely that E-cadherin suppresses p27 expression, because both E-cadherin and p27 generally function as tumor suppressors. In order to proliferate, however, cells require attachment to the extracellular matrix (ECM) via adhesion molecules, such as integrins. When deprived of attachment to the ECM, cells are unable to grow *per se* and in turn p27 levels are increased (18). Similarly, the lack of cell-cell contact retards cell growth. The engagement of E-cadherin in homophilic cell-cell contact may be required for cell growth.

Contact inhibition is widely acknowledged in non-transformed cells as a density-dependent inhibition of cell growth with upregulation of p27 in a monolayer culture, but transformed cells lose this phenomenon to varying degrees (19). Recent evidence has suggested that contact inhibition occurs due to the inactivation of EGFR by the overexpression of E-cadherin (20).

Notably, it has been proved that EGFR is co-localized to adhesive structures with E-cadherin at the early stages of the

formation of cell-cell adhesion complexes, and E-cadherin stimulates the MAPK pathway through the ligand-independent activation of EGFR (2,21). Furthermore, a number of recent studies have demonstrated that mitogenic signals induce p27 degradation (22,23).

The loss of E-cadherin is widely observed in various cancers, especially in advanced cancers with invasion or metastasis to other organs. Thus, the alteration of E-cadherin expression *in vivo* is a critical point of tumor progression. The loss of E-cadherin results in the accumulation of free ß-catenin within the cytoplasm and nucleus, and in turn activates the target gene of proliferation through the Wnt signaling pathway. However, we did not find any significant correlation between ß-catenin and p27 or E-cadherin protein levels by Western blot analysis or immunohistochemistry. The participation of E-cadherin in the Wnt-signaling pathway is still controversial (24).

We propose the relationship between E-cadherin and p27 as illustrated in Fig. 6. In both *in vitro* and *in vivo* proliferating tumor cells, p27 is downregulated because of E-cadherin-induced contact stimulation. In the plateau phase of a monolayer culture, both E-cadherin and p27 are upregulated in a cell density-dependent manner, i.e. contact inhibition. However, in the advanced stage with invasion or metastasis *in vivo*, p27 may be upregulated by the loss of E-cadherin engagement.

We previously reported p27 to be a prognostic marker in renal cell carcinoma; however accumulating evidence has demonstrated a paradoxical increase in p27 expression in a subset of aggressive cancers (25-27). Moreover, very recently an oncogenic function of p27 has also been demonstrated (28-30). Therefore the precise regulatory mechanism of p27 expression and its biological function in tumors await further clarification.

Our results provide new insight into the relationship between cell-cell contact and the cell cycle, and suggest a possible regulatory mechanism of p27 through outside-in signaling pathways from E-cadherin.

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