Changes in E-cadherin associated with cytoplasmic molecules in well and poorly differentiated endometrial cancer

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Summary  E-cadherin function is thought to be impaired in epithelial cancer. To investigate the alterations in E-cadherin associated with cytoplasmic molecules including α-catenin, β-catenin, γ-catenin, p120CAS, and IQGAP1 in various endometrial cancers with different degree of differentiation, we examined the localization and expression of E-cadherin and cytoplasmic molecules in 30 cases of both well and poorly differentiated endometrioid adenocarcinomas, using immunofluorescence and immunoblotting techniques. E-cadherin and cytoplasmic molecules demonstrated linear staining at the cell boundaries in normal endometrium. In all 20 cases with well differentiated adenocarcinomas, α-catenin and IQGAP1 disappeared from the cell adhesive sites, but other cytoplasmic molecules were co-localized with E-cadherin along the cell boundaries. In all 10 cases with poorly differentiated adenocarcinomas, E-cadherin and cytoplasmic molecules accumulated as large aggregates along cell adhesive sites, and the localization of IQGAP1 differed from those of other cytoplasmic molecules. The expression of these molecules in all 20 cases with well differentiated adenocarcinomas decreased or was lost in Triton-insoluble fraction, in comparison with the findings for all cases with normal endometrium or poorly differentiated adenocarcinomas. These results suggested that each alteration in E-cadherin associated with cytoplasmic molecules may play a different role in E-cadherin dysfunction between well and poorly differentiated adenocarcinomas. © 2000 Cancer Research Campaign

Keywords: IQGAP1; α-catenin; E-cadherin; endometrial cancer

During embryonic development and adult life, the cadherin superfamily of cell adhesion molecules is known to be involved in many processes including cell segregation, proliferation, differentiation, and invasive behaviour (Takeichi, 1993). In human cancer, E-cadherin, which is an epithelial cell adhesion molecule, has been recognized as a powerful invasion-suppressor. It has been reported that the function of E-cadherin in zonula adherens junctions is impaired in many kinds of epithelial cancers (Shiozaki et al, 1996; Hirohashi, 1998).

Endometrial cancer, one type of epithelial cancer, is a unique epithelial cancerous state with varying levels of differentiation. In general, the oestrogen and progesterone receptors are expressed in many cases of well differentiated endometrioid adenocarcinomas, but are lacking in poorly differentiated endometrioid adenocarcinomas (Creasman, 1993). In terms of clinicopathological properties, poorly differentiated adenocarcinomas are characterized by deep myometrial invasion, high metastatic ability and a poor clinical prognosis (Kauppila et al, 1982; Creasman, 1993). In contrast, well differentiated adenocarcinomas of the endometrium have a less invasive ability, less metastatic potential, but a more favourable prognosis (Kauppila et al, 1982; Creasman, 1993). Thus, there seem to be distinct differences in cancer cells between well and poorly differentiated adenocarcinomas.

E-cadherin is a homophilic cell adhesion molecule, which depends on the presence of Ca2+ ions. It is a transmembrane protein with five tandemly repeated extracellular domains and a cytoplasmic domain that connects the actin cytoskeleton through a complex with α-, β-, and γ-catenins. E-cadherin and cytoskeletal molecules play a crucial role in cellular morphogenesis and cell regulation, including cell shape and function (Takeichi 1993; Aberle et al, 1996; Drubin and Nelson 1996; Gumbiner, 1996). Protein p120CAS has also been defined as a member of the cadherin-based cell–cell adhesion complex, containing a series of 42 amino acid armadillo repeats, and binds directly to E-cadherin (Daniel and Reynolds, 1995). IQGAP1, as a candidate effector for the Rho family of GTPases, including Rac1 and Cdc42, can also bind β-catenin and E-cadherin (Kuroda et al, 1998). Therefore, E-cadherin function is apparently influenced by the binding of α-catenin, β-catenin, γ-catenin, p120CAS, or IQGAP1 in different cell regulating processes.

To investigate the alterations of E-cadherin associated with cytoplasmic molecules among subtypes of endometrial cancers with varying differentiation, we examined the localization and expression of E-cadherin and cytoplasmic molecules, using surgical specimens from a normal endometrium and malignant endometrial tissues including well and poorly differentiated adenocarcinomas. We found that the localization of α-catenin and IQGAP1 were disturbed in a different manner in well and poorly differentiated adenocarcinomas, compared to the findings for the normal endometrium. Comparing extracted proteins at cell adhesive sites from well differentiated adenocarcinomas, we found remarkable differences in protein expression and protein
modification of E-cadherin and cytoplasmic molecules in Triton-insoluble fraction, compared to that of poorly differentiated adenocarcinomas. These results suggested that changes in E-cadherin associated with cytoplasmic molecules may be involved in the impairment of E-cadherin function in endometrial cancers with varying degrees of differentiation.

**MATERIALS AND METHODS**

**Human tissues**

Fresh specimens of endometrioid adenocarcinomas (20 well differentiated and 10 poorly differentiated adenocarcinomas) were obtained at surgery from 30 Japanese patients with endometrial carcinomas. The clinical features of the two types of endometrial cancers are summarized in Table 1. Normal endometrial tissue specimens were also obtained at surgery for benign disease from 4 Japanese patients in the follicular phase of the menstrual cycle. After dissection, some tissue specimens were embedded in OCT compound (Miles Laboratories, Elkhart, IN), and immediately snap-frozen in liquid nitrogen. Frozen sections were cut on a cryostat to a thickness of 4 μm, mounted on poly-l-lysine-2 coated slides, and either used immediately or stored at −80°C until needed, in order to assess the co-localization of E-cadherin and its associated cytoplasmic molecules using immunofluorescence analysis. The remaining tissue specimens dissected from the same patient were directly frozen in liquid nitrogen and also stored at −80°C, after which proteins were extracted, in order to examine the protein expression of E-cadherin and its associated cytoplasmic molecules using western blotting and tyrosine phosphorylation of β-catenin and p120CAS using immunoprecipitation.

**Immunostaining**

Sections for immunostaining were fixed in PBS (phosphate-buffered saline) with 4% paraformaldehyde and 5% sucrose for one hour at room temperature, washed three times with PBS, and permeabilized for 5 min with 0.4% Triton X-100 in PBS. The sections were next washed three times with PBS, and blocked for 30 min with 5% goat serum in PBS. These sections were incubated overnight with primary antibodies at 4°C, after which proteins were extracted, in order to examine the protein expression of E-cadherin and its associated cytoplasmic molecules using western blotting and tyrosine phosphorylation of β-catenin and p120CAS using immunoprecipitation.

*Table 1* Clinical features in endometrial cancers

|                | G1 (N = 20) | G3 (N = 10) |
|----------------|------------|------------|
| Ages           | 54.2 ± 12.3 (30–72) | 51.3 ± 13.2 (30–66) |
| Stages         |            |            |
| I              | 14         | 5          |
| II             | 4          | 3          |
| III            | 2          | 2          |
| IV             | 0          | 0          |
| Myometrial     |            |            |
| None           | 11         | 1          |
| Invasion       |            |            |
| ≤ 1/2          | 9          | 2          |
| > 1/2          | 0          | 7          |
| Lymph Node Involvement | 1          | 7          |

G1: Well differentiated adenocarcinomas, G3: Poorly differentiated adenocarcinomas.

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British Journal of Cancer (2000) 83(9), 1168–1175
Triton-insoluble components (Miyamoto et al., 1995). The protein concentrations of these two fractions were determined using a modification of the method of Bradford (Pierce Chemical, Rockford, IL, USA) (Bradford, 1976). The extracted protein ratios (mean ± standard deviation) between the Triton-insoluble components and the Triton-soluble components (Triton-insoluble components/Triton-soluble components) were 0.64 ± 0.21 (normal endometrial tissues), 0.60 ± 0.22 (well differentiated adenocarcinomas), and 0.65 ± 0.18 (poorly differentiated adenocarcinomas).

Immunoblotting and immunoprecipitation

An equivalent amount (30 μg for the Triton-insoluble components and 70 μg for the Triton-soluble components) of each fraction was subjected to SDS-PAGE and was analysed by Western immunoblotting. To examine the tyrosine phosphorylation of β-catenin and p120CAS, immunoprecipitation was performed for the protein (1000 μg) of each fraction, by using 5 μg anti-β-catenin and 5 μg anti-p120CAS antibodies, and the precipitated samples were applied to SDS-PAGE and analysed by Western immunoblotting, using an anti-phosphotyrosine antibody, and then the same immunoblot was re-analysed with the appropriate antibodies.

Statistical analysis

Comparisons of co-localization scorings among the three groups were made using the Bonferroni’s t-test. P values < 0.05 were interpreted as indicating a significant difference.

RESULTS

Co-localization of E-cadherin and its associated cytoplasmic molecules including α-catenin, β-catenin, γ-catenin, p120CAS, and IQGAP1

To investigate the co-localization between E-cadherin and its associated cytoplasmic molecules, double immunostaining for E-cadherin and IQGAP1, the three catenins, or p120CAS were examined in normal endometrium, well, and poorly differentiated adenocarcinomas. E-cadherin and its associated cytoplasmic molecules were expressed only in normal endometrial cells, endometrial cancer cells, and endothelial cells of vascular vessels within the tissue specimens. E-cadherin and IQGAP1 co-localized at cell adhesive sites as well as at the regions bordering stromal tissues in normal endometrium (Fig. 1A,B,C). In well differentiated adenocarcinomas, the immunostaining signals for E-cadherin and IQGAP1 revealed large aggregates along the cell boundary (G and H). Co-localization of E-cadherin and IQGAP1 is shown for each of the three groups (C, F and I). Each inset shows a higher-magnification view. Bar, 20 μm.
E-cadherin dysfunction and tumour differentiation

The localization of β-catenin (green) or E-cadherin (red) was determined using fluorescein or Texas-red fluorescence, and immunofluorescence overlap of β-catenin and IQGAP1 was observed as a yellow signal (*). β-catenin and E-cadherin proteins were observed at the zonula adherens junctions in normal endometrium (A and B), well differentiated adenocarcinomas (D and E), and poorly differentiated adenocarcinomas (G and H). Co-localization of β-catenin and E-cadherin is shown for each of the three groups (C, F and I). Each inset shows a higher-magnification view. Bar, 20 μm

to gain more insight into the distribution of each cytoplasmic molecule mediated by E-cadherin, we compared the localization of α-catenin, β-catenin, γ-catenin and p120CAS to IQGAP1 in these two types of endometrial cancers. In well differentiated adenocarcinomas, the signals for α-catenin as well as IQGAP1 were diffusely distributed, without any co-localization inside the cells (Fig. 3A,B,C). In poorly differentiated adenocarcinomas, α-catenin and IQGAP1 were in part localized along cell boundaries, but immunofluorescent signals for α-catenin did not coincide with those of IQGAP1 at all (Fig. 3D,E,F). No morphological difference between IQGAP1 positive and α-catenin positive parts was found in the sections using haematoxylin and eosin staining. The co-localization scorings of the three catenins or p120CAS with E-cadherin, and E-cadherin, the three catenins, or p120CAS with IQGAP1 were shown in Table 2. E-cadherin and IQGAP1 were usually detected in the basolateral regions of the cells in normal endometrium. The three catenins and p120CAS were sometimes localized in the basolateral regions only in normal endometrium. In all 4 cases of normal endometrium, the immunofluorescent signals for α-catenin, β-catenin, γ-catenin, p120CAS, or IQGAP1 revealed a linear accumulation at the cell boundaries, and all the E-cadherin associated with cytoplasmic molecules were positively co-localized with E-cadherin. In all 20 cases of well differentiated adenocarcinomas, the immunofluorescent signals for β-catenin, γ-catenin, or p120CAS indicated thinly broken lines or diffuse bands, and α-catenin or IQGAP1 was diffusely distributed inside the cells. The co-localization scorings of β-catenin, γ-catenin, or p120CAS with E-cadherin were almost the same as those in patients with normal endometrium, whereas the co-localization scorings of α-catenin with E-cadherin and E-cadherin, the three catenins, or p120CAS with IQGAP1 significantly decreased, compared with the scorings in patients with normal endometrium and poorly differentiated adenocarcinoma because of a diffuse distribution of α-catenin and IQGAP1. In all the 10 cases of poorly differentiated adenocarcinomas, the immunofluorescent signals for α-catenin, β-catenin, β-catenin, γ-catenin, p120CAS, or IQGAP1 partly accumulated along the zonula adherens junctions as clumped aggregates, and each co-localization scoring of the three catenins or p120CAS with E-cadherin significantly decreased, compared with those in normal endometrium, although only the co-localization scoring of E-cadherin with IQGAP1 was almost the same percentage as those in patients with normal endometrium. In poorly differentiated adenocarcinoma, the three catenins and p120CAS were distinctly expressed in some of the cancer cells, which had no expression of
The distribution of α-catenin and IQGAP1 in well differentiated adenocarcinomas (G1) and poorly differentiated adenocarcinomas (G3). The localization of α-catenin (green) or IQGAP1 (red) was determined using the fluorescein or Texas-red fluorescence, and immunofluorescence overlap of α-catenin and IQGAP1 was observed as a yellow signal (*). The diffuse co-localization of E-cadherin and its associated cytoplasmic molecules was evident in well differentiated adenocarcinomas.

In each of the 4 cases of normal endometrium in the secretory phase or in menopause, E-cadherin and its associated cytoplasmic molecules were co-localized as a clear line at the zonula adherens junctions, just like a normal endometrium in the follicular phase. In both types of endometrial cancers, the immunofluorescent signals for β-catenin were partly detected in the cytoplasm, but no distinct accumulation of β-catenin in the nucleus was found in the normal endometrium or endometrial cancers.

Table 2  Co-localization of E-cadherin and its associated cytoplasmic molecules

| E-Cadherin (P) | IQGAP1 (P) |
|---------------|------------|
| N (N = 4)     | G1 (N = 20) | G3 (N = 10) | N (N = 4) | G1 (N = 20) | G3 (N = 10) |
|               |            |            |            |            |            |
| E-cadherin (M) | 95.2 ± 4.5 | 96.2 ± 4.5 | 95.4 ± 7.5 | 94.2 ± 3.8 | 15.9 ± 8.2a | 80.2 ± 14.6 |
| α-catenin (M)  | 89.2 ± 4.2 | 15.2 ± 9.5a | 64.5 ± 6.5a | 89.5 ± 4.2 | 5.2 ± 7.2a | 16.2 ± 8.2a |
| β-catenin (M)  | 89.2 ± 4.3 | 82.3 ± 7.0 | 62.5 ± 4.8a | 102 KDa    | 92 KDa      | 102 KDa      |
| γ-catenin (M)  | 87.5 ± 6.2 | 81.3 ± 8.2 | 59.2 ± 4.0a | 170 KDa    | 140 KDa     | 110 KDa      |
| p120CAS (M)    | 90.5 ± 4.2 | 83.2 ± 7.4 | 68.2 ± 7.0a | 170 KDa    | 140 KDa     | 110 KDa      |

Table shows the results for double staining, using monoclonal antibodies (M) and polyclonal antibodies (P). *Significant decrease of co-localization scoring, compared to patients with normal endometrium. N: Normal endometrium, G1: Well differentiated adenocarcinomas, G3: Poorly differentiated adenocarcinomas.
endometrium, one of 5 cases with well differentiated adenocarcinomas, and in 4 of 5 cases with poorly differentiated adenocarcinomas. To confirm the loss of other cytoplasmic molecules associated with E-cadherin, we examined the expression of α-catenin and IQGAP1 in the two fractions. In the Triton-insoluble fraction, α-catenin and IQGAP1 were clearly expressed in all 4 cases with normal endometrium and all 5 cases with poorly differentiated adenocarcinomas, but no expression of α-catenin or IQGAP1 was noted in any of the 5 cases with well differentiated adenocarcinomas. However, α-catenin and IQGAP1 in the Triton-insoluble fraction were distinctly expressed in all tissue specimens (Fig. 4). Table 3 summarizes the expression of E-cadherin and its associated cytoplasmic molecules in each fraction among normal endometrium, well and poorly differentiated adenocarcinomas. In all 4 cases with normal endometrium, E-cadherin, 3 catenins, p120CAS, and IQGAP1 were distinctly expressed in both fractions. Among the 20 cases with well differentiated adenocarcinomas, the expression of E-cadherin was found in 5 cases, and in a few cases, the expression of β-catenin, γ-catenin, or p120CAS was noted in Triton-insoluble fraction. However, the expression of α-catenin or IQGAP1 in Triton-insoluble fraction was detected in none of the 20 cases. In all 10 cases of poorly differentiated adenocarcinomas, the expression of E-cadherin, α-catenin, γ-catenin, p120CAS, or IQGAP1 was distinctly found, and the expression of β-catenin was detected in 9 of 10 cases in Triton-insoluble fraction. Each expression of E-cadherin, the three catenins, p120CAS, or IQGAP1 was distinctly found in Triton-soluble fraction extracted from all the cases including the 4 with normal endometrium, 20 cases with well differentiated adenocarcinomas, and 10 cases with poorly differentiated adenocarcinomas.

**DISCUSSION**

Cell adhesive properties mediated by E-cadherin are regulated by cytoplasmic molecules including catenins, p120CAS, and IQGAP1, as well as by small GTPases, such as Rac1, Cdc42, or Rho (Braga et al, 1997; Kuroda et al, 1997; Takaishi et al, 1997; Jou and Nelson 1998). Regarding E-cadherin and its associated cytoplasmic molecules, adhesiveness at cell adhesion sites is enhanced, when E-cadherin links to α-catenin (Nagafuchi et al,
Normal protein modification such as tyrosine-phosphorylation of β-catenin or p120CAS induces a loss of cell adhesion mediated by E-cadherin (Matsuyoshi et al, 1992; Behrens et al, 1993; Kinch et al, 1995). On the other hand, IQGAP1 mediates the dissociation between E-cadherin and α-catenin (Kuroda et al, 1998). In human cancers, this loss of E-cadherin function has been reported to involve several different mechanisms: the down-regulation of E-cadherin expression and gene mutation, abnormality or deletion of catenins including α-catenin, and abnormal biochemical modification of cytoplasmic molecules such as tyrosine-phosphorylation of β-catenin or p120CAS (Vlemincx et al, 1991; Takeichi, 1993; Mareel et al, 1994; Kinch et al, 1995; Shiozaki et al, 1996; Hirohashi, 1998). However, little is known about changes in the localization, expression, or protein modification of E-cadherin and its associated cytoplasmic molecules in epithelial cancers with different degrees of differentiation.

Our evidence obtained in the present study shows that in the normal endometrium, the zonula adherens junctions seem to be maintained by the accumulation of E-cadherin and its associated cytoplasmic molecules including α-catenin, β-catenin, γ-catenin, p120CAS, and IQGAP1, at cell adhesive sites. In well differentiated adenocarcinomas, the complete loss of α-catenin and IQGAP1, the decrease in expression of E-cadherin, β-catenin, γ-catenin, and p120CAS, and the reduction of tyrosine-phosphorylation of β-catenin at cell adhesive sites were found, compared with findings in normal endometrium, although E-cadherin and its associated cytoplasmic molecules were expressed in Triton-soluble fraction. Taken together, these results suggest that the decrease of E-cadherin and loss of α-catenin at cell adhesives sites may induce impairment of cell adhesiveness mediated by E-cadherin in well differentiated adenocarcinomas. Since the binding of IQGAP1 to E-cadherin induces a dissociation of cell adhesive sites, the disappearance of IQGAP1 on zonula adherens junctions might compensate for the impairment of the E-cadherin function. Tyrosine-phosphorylation of β-catenin may contribute to the maintenance of E-cadherin function because β-catenin at cell boundaries was tyrosine-phosphorylated in normal endometrium. However, it remains to be elucidated as to whether or not the decrease of tyrosine-phosphorylation of β-catenin has any effect on E-cadherin function.

In some cancers including gastric and colorectal cancer, no significant correlation was observed between E-cadherin expression and distant metastasis (Kinesella et al, 1992; Oka et al, 1992; Dorudi et al, 1993; Mayer et al, 1993). Oka et al reported that the majority of primary tumours and all tumours in live metastatic sites of gastric cancer expressed the E-cadherin protein (Oka et al, 1992). Previous studies suggested the possibility that tumour cells express immunoreactive E-cadherin that does not function normally (Oka et al, 1992; Becker et al, 1994; Matsui et al, 1994). In poorly differentiated adenocarcinoma, the abnormal complexes such as E-cadherin and IQGAP1 or E-cadherin and other cytoplasmic molecules were formed at different cell adhesive sites, and the strong tyrosine phosphorylation of β-catenin in Triton-insoluble fraction was observed. These results suggest that E-cadherin might fail to function because of a complete disruption to the cytoplasmic machinery even though E-cadherin was distinctly expressed at cell adhesive sites. On the other hand, the following things are also speculated by our findings: in some cancer cells from tissues with poorly differentiated adenocarcinomas, the aggressive accumulation of IQGAP1 bound to E-cadherin may induce the disappearance of catenins and p120CAS from the cell adhesive sites and may mediate the dissociation of E-cadherin at the zonula adherens. In other cancer cells, the loss of IQGAP1 at cell adhesive sites may induce the tight binding of the catenins and p120CAS to E-cadherin, and may enhance the function of E-cadherin at the zonula adherens. In poorly differentiated adenocarcinomas, these phenomena would happen repeatedly within the cells, as well as in the tissues. In addition, the tyrosine-phosphorylation of β-catenin seemed to increase in poorly differentiated adenocarcinomas, compared with that of normal endometrium. As a result, it is possible that tyrosine-phosphorylation of β-catenin at cell adhesive sites contributes to the impairment of E-cadherin function. Thus, E-cadherin function might be impaired by completely deviating from normal cytoplasmic machinery. However, the molecular mechanism as well as the biological meaning of our findings remains unclear.

The tyrosine-phosphorylation of P120CAS had little influence on the development of endometrial cancers because there was no significant difference in the tyrosine-phosphorylation of P120CAS among patients with normal endometrium, well, and poorly differentiated adenocarcinomas. In endometrial cancer, the mutated protein of β-catenin is localized within the cells, in the nucleus as well as the cytoplasm (Kobayashi et al, 1999). In our study, immunofluorescent signals for β-catenin were partly detected in the cytoplasm as a diffuse distribution. However, a distinct accumulation of β-catenin only in the nucleus was not observed in the tissue samples. It is speculated that the mutation of β-catenin could be detected in some cases, which had a diffuse distribution of β-catenin in the cytoplasm.

The adhesion function mediated by E-cadherin is regulated by E-cadherin and its associated cytoplasmic molecules, including α-catenin, β-catenin, γ-catenin, p120CAS, IQGAP1, and by small GTPases, and is possibly associated with such tumour suppressor genes as APC and DCC or other unknown molecules (Gumbiger 1996; Braga et al, 1997; Kuroda et al, 1997; Morin et al, 1997; Ribinfeld et al, 1997; Takaishi et al, 1997; Jou and Nelson 1998). In this study, we showed that each change in cytoplasmic molecule bound to E-cadherin may be involved in endometrial cancers with varying degrees of differentiation, which are also thought to have different tumour characteristics. Therefore, an impairment of E-cadherin function in epithelial cancers may be induced by a variety of molecular mechanisms.
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