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Immunohistochemical evaluation of E-cadherin adhesion molecule expression in human gastric cancer

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Summary. E-cadherin (ECD) is one of subclasses of the cadherin family which plays a major role in the maintenance of intercellular adhesion in epithelial tissues. An immunohistochemical study of ECD expression was performed on gastric adenocarcinoma from 103 patients using our monoclonal antibody for human ECD (HECD-1). ECD was strongly expressed in normal gastric epithelium without exception; however, various staining patterns were observed in cancer tissues. The frequency of tumours with preserved ECD expression (Pre-type) and reduced ECD expression (Rd-type) was 42% and 58% respectively. Tumours with a high frequency of Rd-type expression particularly included: undifferentiated tumours (85%, 46/54), Borrmann's type 4 (90%, 9/10), tumours larger than 2.6 cm in diameter (65%, 53/81), tumours invading beyond the subserosa layer (78%, 46/59), and tumours with infiltrative growth (87%, 41/47). Furthermore, the frequency of Rd-type expression in cases with peritoneal dissemination (82%, 9/11) or lymph node metastasis (73%, 43/59) was significantly higher than that in cases without dissemination or metastasis. These results suggest that ECD might play a key role in the genesis of histological differentiation, and that the reduction of ECD expression may affect the mode of invasion and metastasis of human gastric cancer cells.

Key words: Gastric cancer – E-cadherin – Immunohistochemistry – Cancer invasion

Introduction

Cadherins are the family of transmembrane glycoproteins responsible for calcium-dependent intercellular adhesion. They were identified by preparing antibodies capable of blocking intercellular adhesion between mouse teratocarcinoma cells (Yoshida and Takeichi 1982). cDNA cloning of various cadherins has been reported (Hatta et al. 1988; Nose et al. 1987), and they represent a gene family completely different from that of integrin and immunoglobulin superfamily (Takeichi et al. 1988). Cadherins produce the strong intercellular connections by homophilic interaction in the presence of calcium, and the inactivation of the other adhesion systems has little effect on cell-cell adhesion when cadherins are functioning (Yoshida et al. 1984). Thus, cadherins are crucial for the mutual association of vertebrate cells (Takeichi 1991). Cadherins are divided into subclasses such as epithelial type E-cadherin (ECD) (Nagafuchi et al. 1987), neural type N-cadherin (Hatta and Takeichi 1986), placental type P-cadherin (Nose et al. 1987), L-CAM and other subtypes (Gallin et al. 1987; Heimark et al. 1990). Each type of cadherin subclass specifically interacts with its identical type (Nose et al. 1990). ECD molecules which were distributed in epithelial tissues have been reported to be responsible for organogenesis and morphogenesis of these tissues (Takeichi 1988).

The process of tumour metastasis occurs by way of a complex series of sequential steps (Nicolson 1988) that are influenced by various properties of tumour cells. Since the detachment and attachment of tumour cells might affect the initial and subsequent steps in the metastatic process, the mechanisms of cell-cell adhesive interactions and adhesion to extracellular matrix or endothelium could play an important role (Kramer and Nicolson 1979; Terranova et al. 1982). However, the molecular basis of the mutual adhesiveness of cancer cells has not yet been clarified in vivo.

It is expected from biological behaviour that ECD play major roles in intercellular physical adhesion of cancer cells. The suppression of ECD expression or functional activity might trigger the release of cancer cells from the primary lesion. Therefore, it is of interest to study how ECD molecules are involved in the invasion and metastasis of cancer cells. We demonstrated the existence of impaired ECD expression in human cancer tissues in previous reports (Shiozaki et al. 1991; Takeichi

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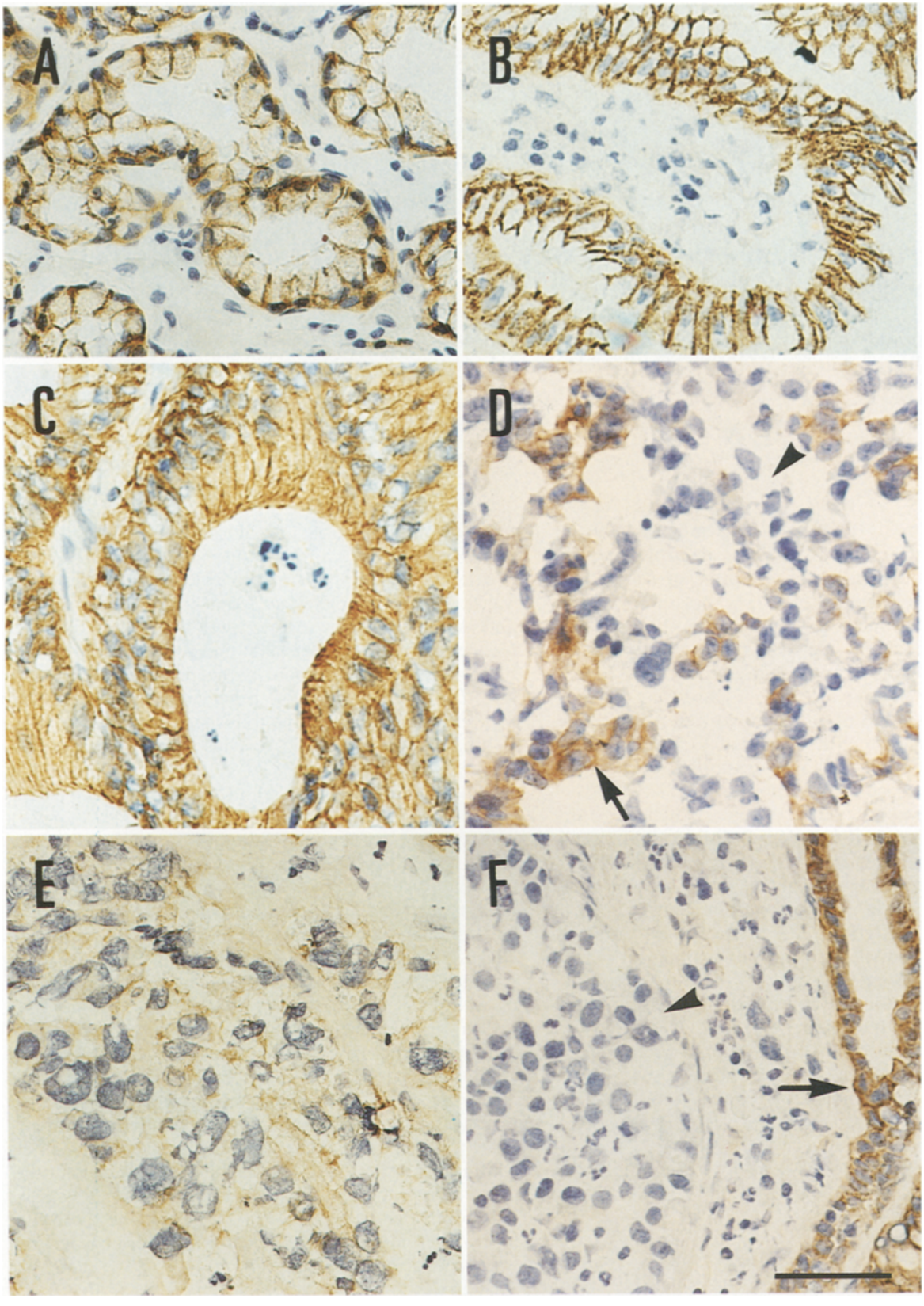

Fig. 1 A–F. Immunohistochemical reactivity of E-cadherin in human gastric carcinoma. **A, B** Normal gastric foveola and glands. **C** Preserved ECD expression (+): well-differentiated adenocarcinoma of which ECD expression is as strong as that of normal epithelium. **D** Heterogeneously reduced expression (±): poorly differentiated adenocarcinoma which is a mixture of the cells with different ECD expression; *arrow* and *arrowhead* indicate the ECD-positive and ECD-negative cell respectively. *E* Uniformly reduced expression (−): ECD expression of all the cells in the tumour was recognizable but more weakly marked than that of the cells in normal epithelium. **F** (−): ECD expression of all cells in the tumour was negative. *Arrowhead* indicates ECD-negative cells, and *arrow* indicates the cells in non-cancerous epithelium. PFA-fixed, frozen sections; ABC; haematoxylin counterstain, ×100. *Scale bar* represents 50 μm

1991); however, clinicopathological investigation was not performed in a sufficient number of patients. In order to reveal these subjects, we studied ECD expression in a more extended series of human gastric cancer tissues of primary and metastatic sites using the immunohistochemical staining with our specific monoclonal antibody (mAb) (Shimoyama et al. 1989).

Materials and methods

The specimens were surgically obtained from 103 consecutive patients with gastric adenocarcinoma from December 1989 to May 1991. There were 65 men and 38 women with a mean age of 59.7 years (range, 32–89 years). No patients had received anticancer therapy before operation. Samples were taken from the representative cancerous lesions including adjacent mucosa. Depending upon the size of the tumour, one to five (mean, 2.48) samples were obtained. Metastatic lymph nodes from 37 patients and 3 liver metastatic lesions were also obtained at surgery and examined.

Immunostaining for ECD was performed by the avidin-biotin-peroxidase complex method (Hsu et al. 1981) as described previously (Shiozaki et al. 1991). In brief, the fresh samples were immediately frozen by dry ice acetone, and were cut into sections 4 μm thick. Then the sections were fixed with 3.6% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Anti-human ECD (HECD-1) was applied at the dilution of 1:1000 and incubated overnight at 4° C, sequentially followed by biotinylated goat anti-mouse IgG and avidin combined in vitro with biotinylated horseradish peroxidase (Vectastain ABC kit, Vector). The colour was developed with the diaminobenzidine supplemented with 0.02% hydrogen peroxide for 4 min.

The intensity of ECD staining for cancer cells was compared with that of the cells in non-cancerous epithelium in the same sample (Fig. 1 A, B). The cancer cells whose ECD staining was strong as normal epithelial cells were defined as ECD positive. The cancer cells which show much weaker or negative staining were defined as ECD-negative. The grade of ECD expression of the tumours was semi-quantitatively evaluated according to the proportion of ECD-positive cells. When more than 90%, between 10% and 90%, and less than 10% of the cancer cells were ECD positive, the tumours were evaluated as (+), (±), and (−), respectively (Fig. 1 C–F). The ECD (+) tumours were termed as preserved ECD expression (Pre-type), and the others (± and −tumours) were termed reduced ECD expression (Rd-type).

In order to confirm our evaluation of immunohistochemical staining, we performed immunoblot analysis on the representative samples in each staining type as described previously (Nose and Takeichi 1986). In brief, samples were separated by SDS-PAGE using 7.5% polyacrylamide gels. After electrophoresis, proteins were incubated with mAb HECD-1, followed by incubation with ^{125}I-labelled anti-mouse IgG (Amersham). Radiolabelled electrophoretic bands were visualized by autoradiography. Total cellular proteins applied to each lane were adjusted to be equal using Biorad protein assay kit.

A consecutive section from each specimen was stained with haematoxylin and eosin for histological evaluation. The clinicopathological terminology is derived from the general rules proposed by the Japanese Research Society for Gastric Cancer (1981). Assessment of the presence of the metastases was performed using the procedures including imaging, surgical exploration and/or histological confirmation. There were 15 primary tumours consisting of the mixture of histologically different tumour components. In these tumours, the correlation between ECD expression and histological types was evaluated for each tumour component.

The statistical analysis was performed comparing Pre-type versus Rd-type by the chi-square test.

Results

All the normal gastric epithelial cells strongly expressed ECD molecules at the cell-cell boundaries (Fig. 1 A, B). Non-epithelial cells did not express ECD molecules. Of the 103 primary tumours examined, 43 tumours (42%) and 60 tumours (58%) were classified into Pre-type and Rd-type, comprising 38 (±) and 22 (−) tumours (Table 1) (Fig. 1 C–F).

Figure 2 shows the results of immunoblot analysis. Immunoblotting of normal gastric epithelium reveals four bands (lane 1) and the darkest band was detected at 124 kDa, which corresponds to that of full-size ECD. The intensities of the bands at the 124 kDa position in tumour samples were correlated to immunohistochemical evaluation for ECD expression.

Table 1 shows the relationship of ECD expression to gross appearance and tumour size. In 25 infiltrative type, i.e. Borrmann's type 3 and 4, 19 (76%) were evaluated as Rd-type. Furthermore, the frequency of Rd-type was significantly higher in Borrmann's type 4 (90%, 9/10) than in any other type of tumours ($P<0.01$). A significant correlation was found between tumour size and ECD expression. The frequencies of Rd-type in tumours of smaller than 2.5 cm, 2.6–5.0 cm and larger than 5.1 cm were 32%, 51%, and 77%, respectively. The larger the tumours, the higher were the frequencies of Rd-type.

The relationship between ECD expression and histological findings is shown in Table 2. The frequency of Pre-type was high in papillary adenocarcinoma (75%) and tubular adenocarcinoma (70%). In the 18 differentiated type tumours of Rd-type, the majority were of the (±) (94%, 17/18), and only one (6%) was of the (−). On the other hand, Rd-type was observed more

Table 1. Relationship between gross appearance and E-cadherin (ECD) expression

	No.	Pre-type	Rd-type			*P* value
		(+)	(±)	(−)	Subtotal	
Total	103	43 (42%)	38	22	60 (58%)	
Gross appearance[a]						
type-0	27	18 (67%)	4	5	9 (33%)	<0.01
1	5	3 (60%)	2	–	2 (40%)	
2	24	9 (38%)	13	2	15 (62%)	
3	15	5 (33%)	5	5	10 (67%)	
4	10	1 (10%)	7	2	9 (90%)*	
5	22	7 (32%)	7	8	15 (68%)	
Tumour size[b]						
~2.5cm	22	15 (68%)	3	4	7 (32%)	<0.01
2.6~5.0cm	37	18 (49%)	9	10	19 (51%)	
5.1~ cm	44	10 (23%)	26	8	34 (77%)	

[a] Type 0, mucosal and submucosal cancer; type 1–4 corresponding to Borrmann's classification: type 1, polypoid; type 2, ulcerating circumscribed; type 3, ulcerating infiltrative; type 4, diffusely infiltrative; type 5, unclassified

[b] Maximum diameter of tumour

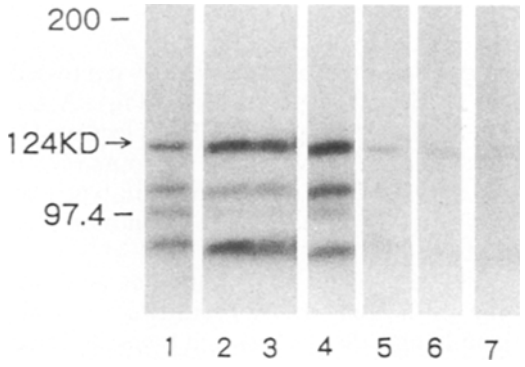

Fig. 2. Immunoblot analysis of ECD expression detected by mAb HECD-1: *lane 1*, normal gastric epithelium; *lanes 2, 3*, primary tumour of differentiated carcinoma classified as (+) with immunohistochemical evaluation; *lane 4*, primary tumour of poorly differentiated carcinoma classified as (+). A strong band for ECD appeared at the 124 kDa position in these samples. *Lanes 5, 6*, primary tumours classified as (±); the bands were faint. *Lane 7*, primary tumour regarded as (−); the bands were totally absent. Full-size ECD is indicated at 124 kDa by *arrow*, and the other three bands with lower molecular weight are considered to represent degraded productions of the molecule

Table 2. Relationship between histological findings and ECD expression

	No.	Pre-type	Rd-type			*P* value
		(+)	(±)	(−)	Subtotal	
Histological type[a]						
pap	8	6 (75%)	2	–	2 (25%)	<0.01
tub	53	37 (70%)	15	1	16 (30%)	
por	37	3 (8%)	15	19	34 (92%)	
sig	17	5 (29%)	7	5	12 (71%)	
muc	3	–	2	1	3 (100%)	
Depth of invasion[b]						
m, sm	27	18 (67%)	3	6	9 (33%)	<0.01
pm	17	12 (71%)	3	2	5 (29%)	
ss, se, si	59	13 (22%)	32	14	46 (78%)	
Growth pattern[c]						
INFα	17	15 (88%)	2	–	2 (12%)	<0.01
INFβ	39	22 (56%)	15	2	17 (44%)	
INFγ	47	6 (13%)	21	20	41 (87%)	

[a] pap, Papillary adenocarcinoma; tub, tubular adenocarcinoma; por, poorly differentiated adenocarcinoma; sig, signet ring cell carcinoma; muc, mucinous carcinoma

[b] m, sm, pm and ss, spread to mucosa, submucosa, muscularis propria and subserosa, respectively; se, exposing on the serosal surface; si, infiltrating the neighbouring tissue

[c] INFα, grows expansively; INFβ, intermediate between α and γ; INFγ, grows infiltratively

frequently in poorly differentiated adenocarcinoma (92%), and signet-ring cell carcinoma (71%). Thus, the frequency of Rd-type was significantly higher in undifferentiated type tumours (85%, 46/54) than in differentiated type tumours (30%, 18/61) ($P<0.01$). However, we found 8 (15%) Pre-type tumours among undifferentiated type tumours (Fig. 3C; Fig. 2, lane 4).

As shown in Table 2, in 27 early tumours with mucosal or submucosal invasion, 18 (67%) were evaluated as Pre-type and 9 (33%) were of the Rd-type. The frequency of Rd-type in tumours with proper muscle invasion or with extension beyond subserosa was 29% and 78%, respectively. Thus, the Rd-type was significantly more frequent in tumours which invaded beyond subserosa than in less extensive tumours ($P<0.01$).

Table 2 also shows the relationship of ECD expression to the invasion pattern of cancer cells. Fifteen (88%) of 17 tumours with expansive growth pattern (INFα) were evaluated as Pre-type, whereas 41 (87%) of 47 tumours with infiltrative growth pattern (INFγ) were determined as Rd-type. The frequencies of (−) in INF α, β and γ were 0%, 5% (2/39) and 43% (20/47) respectively. Thus, the frequency of Rd-type was significantly higher in INFγ tumours than in INFα and β tumours ($P<0.01$).

The results concerning metastasis are shown in Table 3. The frequency of Rd-type in tumours with numerous metastases to the distant peritoneum [P_3] was 100% (6/6), and this value was significantly higher than in tumours without peritoneal dissemination (P_0) (55%, 51/92) ($P<0.05$).

The frequency of Rd-type in tumours with positive lymph node metastasis was 73% (43/59), and this value was significantly higher than in node-negative tumours (39%, 17/44) ($P<0.01$). Furthermore, the Rd-type was more frequent in N2,3 and N4 tumours (83, 71 and 100%, respectively) than in N0 or N1 tumours. Table 4 shows the correlation between lymph node metastasis and ECD expression in the superficial or advanced tumours. Among early tumours, the frequency of Pre-type was high (74%, 17/23) in N0 tumours, whereas the frequency of Rd-type was high (75%, 3/4) in node-positive tumours (not significantly). Among advanced tumours, the frequency of Rd-type was significantly higher in extensively metastatic (N2,3,4) tumours (88%, 14/16) when compared with that in weakly metastatic (N0,1) tumours (42%, 14/33) ($P<0.01$).

We found 8 patients with haematogenic metastasis. Four (57%) of 7 tumours with liver metastases were evaluated as Pre-type, and 3 (43%) were of the (±) in primary lesions. Bone metastasis was found in one patient in which primary tumour was evaluated as (−). Figure 3B shows ECD expression of tumour embolus which is regarded as (±).

As shown in Table 5, we investigated the ECD expression of lymph node metastases in 37 patients, in comparison with that of each primary lesion. In 22 (59%) tumours, ECD was expressed in the same pattern as the individual primary lesion. However, in 22 patients with the (±) primary lesion, 13 (59%) metastatic lymph nodes were evaluated as Pre-type (Fig. 3A). The metastatic liver lesions were also examined in 3 patients, of which one primary tumour was evaluated as ECD (±), and two tumours were of the Pre-type. All of their metastatic liver lesions were evaluated as Pre-type (data not shown).

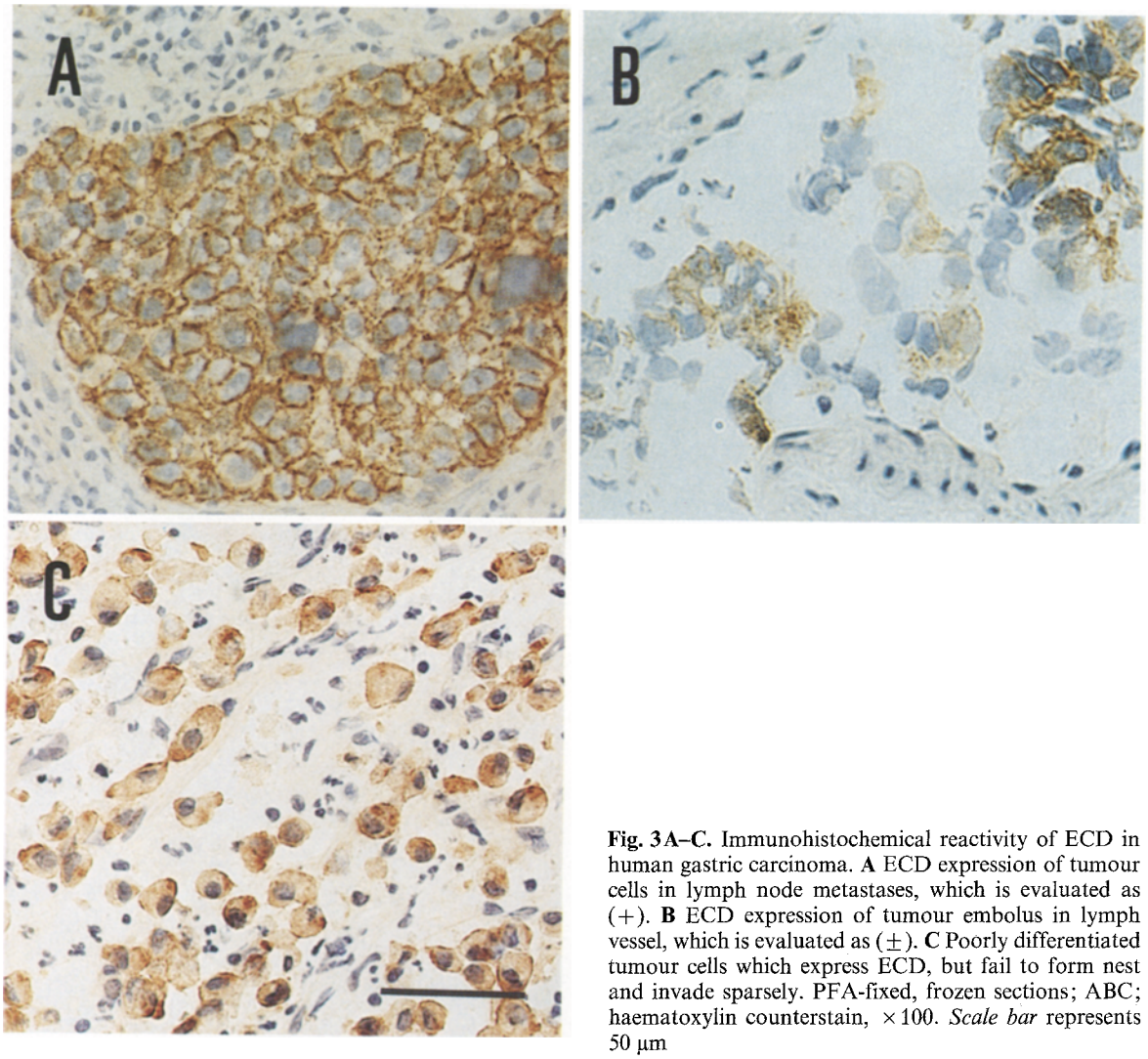

Fig. 3A–C. Immunohistochemical reactivity of ECD in human gastric carcinoma. **A** ECD expression of tumour cells in lymph node metastases, which is evaluated as (+). **B** ECD expression of tumour embolus in lymph vessel, which is evaluated as (±). **C** Poorly differentiated tumour cells which express ECD, but fail to form nest and invade sparsely. PFA-fixed, frozen sections; ABC; haematoxylin counterstain, ×100. *Scale bar* represents 50 μm

There was no significant relationship between ECD expression and age, sex or location of the tumours.

Discussion

In the present study, we found various patterns of impaired ECD expression in gastric cancer tissues, and the frequency of Rd-type tumours was 58%. This result indicates the presence of atypical ECD-related intercellular adhesiveness in gastric cancer cells.

A close correlation has been found between the degree of histological differentiation and ECD expression. ECD expression was frequently (70%) preserved in differentiated tumours, while most undifferentiated tumours (85%) showed reduced expression. Furthermore, we found that ECD-positive cells form clusters or glandular structures, but ECD-negative tumour cells did not (Fig. 1D). These observations indicate that ECD expression on tumour cells could play some role in the genesis of histological types of gastric cancer. Recent in vitro experiments, showing that the cell morphology and the establishment of cell polarities were largely affected by the ECD system, are consistent with our results (Atsumi and Takeichi 1980; Matuzaki et al. 1990; McNeill et al. 1990; Nagafuchi et al. 1987). However, some undifferentiated carcinomas, although their frequency was not so high (15%, 8/54), expressed ECD but were sparsely invasive, not forming close contacts (Fig. 3C). This finding indicates that the ECD is not functioning in these cancer cells. To account for this observation, the following possibilities can be inferred: impaired cadherin molecule with an abnormality in the portion involved in functional regulation (Jaffe et al. 1990; Nagafuchi and Takeichi 1988), or impaired association of cadherins with cytoplasmic components (Nelson et al. 1990; Ozawa et al. 1990).

The frequency of Rd-type expression in tumours of larger size, with deeper invasion, or more infiltrative growth was significantly higher compared with their counterparts. These results suggest that cancer progression may accompany the loss of ECD expression in the course of tumour growth, or that the down-regulation of ECD expression may affect the mode of cancer inva-

Table 3. Relationship between metastasis and ECD expression

	No.	Pre-type	Rd-type			*P* value
		(+)	(±)	(−)	Subtotal	
Peritoneal dissemination[a]						
P0	92	41 (45%)	32	19	51 (55%)	<0.05
P1	5	2 (40%)	2	1	3 (60%)	
P2,3	6	–	4	2	6 (100%)	
Lymph node metastasis[b]						
N0	44	27 (61%)	7	10	17 (39%)	<0.01
N1	19	10 (53%)	7	2	9 (47%)	
N2	23	4 (17%)	15	4	19 (83%)	
N3	7	2 (29%)	4	1	5 (71%)	
N4	10	–	5	5	10 (100%)	
Distant metastasis						
No metastasis	95	39 (41%)	35	21	56 (59%)	NS
Liver metastasis	7	4 (57%)	3	–	3 (43%)	
Bone marrow metastasis	1	–	–	1	1 (100%)	

[a] P0, no dissemination; P1, dissemination to the adjacent peritoneum; P2 and P3, several and numerous metastases to distant peritoneum respectively
[b] N0, no metastasis; N1,2 and 3, metastasis to primary, secondary and tertiary sphere of regional lymph nodes ; N4, metastasis to distant lymph nodes located beyond tertiary lymph nodes

Table 4. Relationship between lymph node metastasis and ECD expression in early (m, sm) and advanced (pm, ss) tumours

	No.	Pre-type	Rd-type			*P* value
		(+)	(±)	(−)	Subtotal	
Early tumours (m, sm)						
N0	23	17 (74%)	1	5	6 (26%)	NS
N1	3	1 (33%)	2	–	2 (67%)	
N2	–	–	–	–	–	
N3,4	1	–	–	1	1 (100%)	
Advanced tumours (pm, ss)						
N0	19	10 (53%)	5	4	9 (47%)	<0.01
N1	14	9 (64%)	4	1	5 (36%)	
N2	12	2 (17%)	8	2	10 (83%)	
N3,4	4	–	3	1	4 (100%)	

Table 5. Relationship between ECD expression in lymph node metastases and in primary lesion

		No.	ECD expression in lymph node metastases		
			(+)	(±)	(−)
ECD expression in primary lesion	(+)	9	8	1	–
	(±)	22	13	9	–
	(−)	6	–	1	5
No.		37	21	11	5

sion. Behrens et al. (1989) demonstrated the association between loss of cadherin function and acquisition of invasive capacity, using their antibodies and transfection experiments (Frixen et al. 1991). Thus, our results indicate that a similar event could occur in human gastric cancer in vivo; the cancer cells with preserved ECD expression tend to grow expansively, but the cancer cells with impaired ECD expression grow in an infiltrative manner.

ECD expression was more frequently impaired in the tumours exhibiting extensive dissemination to peritoneum than the tumours without dissemination. This result is consistent with the speculation that the inhibition of ECD activity may enhance the release of cancer cells from the exposed tumour sites into the peritoneal cavity. Concerning lymph node metastasis, the frequency of Rd-type in extensively metastatic tumours was significantly higher than that in tumours without metastasis. This relationship was also significant when analysed according to the depth of invasion. Hashimoto et al. (1989), using murine ovarian tumour sublines, reported that cells of the weakly metastatic sublines strongly expressed

ECD, whereas cells in the highly metastatic sublines were reduced and heterogeneous. Their report and our results suggest that capability for metastasis of cancer cells to lymph nodes may correlate with their reduced ECD expression, and that the impaired ECD-mediated mutual connection of cancer cells is considered to be a prerequisite for metastasis.

However, 4 (57%) of 7 primary tumours with liver metastasis and all 3 metastatic lesions in the liver were evaluated as Pre-type. Moreover, the ECD expression in the lymph node metastasis was preserved in more than half (21/37) of the cases examined in this study. This incongruity of ECD-expressing tumour cells being capable of metastasizing could be explained by following hypotheses.

1. Cadherin gene expression could be unstable in some tumours (Hashimoto et al. 1989), and ECD expression might locally or temporally diminish, triggering the detachment of certain cell populations so as to induce their metastasis. At the implantation sites, these same cells may again express ECD, or only ECD-positive cells may finally implant. In the case of liver metastasis (Middelkoop et al. 1985; Nicolson 1988), it would be favourable for the ECD-positive cells to attach to hepatocytes which express abundant ECD, and enable them to survive in their new microenvironment.

2. Some tumour cells express ECD but do not function as mentioned above. Since cadherin function is controlled by the cytoplasmic machinery and some intracellular physiological changes linked with malignant transformation could disorganize this machinery, they might cause the destabilization of cadherin-mediated cell adhesion.

Other factors (e.g. motility factors) which can mobilize cancer cells without affecting ECD expression should also be considered (Jounanneau et al. 1991). More detailed analyses are necessary to confirm these speculations.

Recently, Shimoyama and Hirohashi (1991) reported the expression pattern of cadherins in 54 gastric cancer tissues, but the relationship of ECD to clinicopathological factors has not yet been clearly defined. Our results suggest that ECD plays some role in the genesis of histological differentiation, and that the down-regulation of ECD expression may affect the mode of invasion and lymph node metastasis of human gastric cancer cells. Although further studies are required to confirm our speculations with regard to ECD involvement in metastasis, these data provide some encouraging ideas on the molecular basis of tumour invasion and metastasis.

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