Jpn. J. Cancer Res. **89**, 829–836, August 1998

Expression of Cadherin-Catenin Cell Adhesion Molecules, Phosphorylated Tyrosine Residues and Growth Factor Receptor-tyrosine Kinases in Gastric Cancers

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Tyrosine phosphorylation of β-catenin, an intracytoplasmic E-cadherin-binding protein, has been shown to disrupt the cadherin-mediated cell adhesion system *in vitro*. In order to investigate the relationships of expression and tyrosine phosphorylation of cadherin-catenin molecules and expression of growth factor receptor-tyrosine kinase with loose cell-to-cell adhesion, immunohistochemical staining for E-cadherin, α- and β-catenin, phosphorylated tyrosine residues and tyrosine kinase receptors, including *c-erbB-2*, epidermal growth factor-receptor (EGF-R), *c-met* and K-*sam*, in 17 undifferentiated- and 10 differentiated-type human gastric cancers was performed. Loss or reduced expressions of E-cadherin and α- and β-catenin (11, 11, 10 cancers, respectively) were observed in the former, but not the latter. Diffuse cytoplasmic staining of E-cadherin, α- and β-catenin and phosphotyrosine residues was observed frequently in the undifferentiated-type cancers. The cytoplasmic localization of phosphotyrosine residues in undifferentiated-type cancers was correlated significantly with K-*sam* expression (P<0.01) and diffuse cytoplasmic staining of E-cadherin (P<0.05) and β-catenin (P<0.05). Expression of K-sam protein was detected significantly more frequently in undifferentiated- (6/17; P<0.05) than differentiated-type adenocarcinomas whereas the converse applied to *c-erbB-2* expression (8/10 of the latter, P<0.05). Tyrosine phosphorylation of β-catenin was directly confirmed in the protein extracts of one undifferentiated-type gastric cancer. These data indicate that alteration of tyrosine phosphorylation status associated with K-*sam* expression may cause the cytoplasmic distribution of cadherin-catenin molecules and loose cell-cell adhesion in undifferentiated-type gastric cancers.

Key words: Tyrosine phosphorylation — β-Catenin — Cadherin-mediated cell adhesion — Undifferentiated gastric cancer

The cadherins are a family of cell membrane glycoproteins that mediate tight adhesion between cells, and E-cadherin is the major cadherin molecule expressed by epithelial cells.[1] Cadherins form complexes with cytoplasmic proteins called catenins, of which there are three different proteins, α- and β-catenin and plakoglobin (γ-catenin).[2,3] The cadherin-mediated cell adhesion system has been shown to act as an "invasion suppressor system" in cancer cells *in vitro*.[4–6] Structural abnormalities of E-cadherin and α- and β-catenin caused by gene mutations were shown to disrupt E-cadherin-mediated intercellular adhesion and resulted in loose cell-to-cell adhesion in cancer cells.[7,8] CpG methylation of the promoter region of E-cadherin gene has also been proven to suppress E-cadherin expression in cancer cells.[9,10] Another reported mechanism for the dysfunction of the cadherin cell adhesion system is tyrosine phosphorylation of β-catenin.[11] The addition of growth factors, epidermal growth factor (EGF) and hepatocyte growth factor, to cultured human gastric cancer cells resulted in rapid dissociation of cancer cells and tyrosine phosphorylation of β-catenin.[12] We and another group found that β-catenin was associated with receptor-type tyrosine kinases, *c-erbB-2* and the EGF receptor (EGF-R), in several cancer cell lines.[13–15] These data indicate that activation of growth factor receptors in cancer cells may cause cancer cell dissociation through β-catenin tyrosine phosphorylation.

The characteristic histological features of undifferentiated-type gastric cancers are that most of the cancer cells do not form glands and show marked scattered cell growth with loose cell-to-cell adhesion. Loss and reduced expression of E-cadherin and catenins may be a consequence of genetic alterations,[7,16,17] or changes in the methylation state of the promoter region of the E-cadherin gene in cancer cells, as reported previously.[9] However, immunohistochemical studies revealed that about half of the undifferentiated-type cancers showing loose cell-cell adhesion clearly expressed cadherin-catenin cell adhesion molecules. The mechanisms responsible for dysfunction of cadherin-mediated cell adhesion in this type of gastric cancer have not yet been elucidated. In order to clarify if tyrosine phosphorylation of β-catenin causes the loose cell-to-cell adhesion in undifferentiated-type gastric cancers, the expression and localization of growth factor

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receptor-tyrosine kinases and phosphotyrosine residues were compared with those of cadherin-catenin cell adhesion molecules using immunohistochemical techniques.

MATERIALS AND METHODS

Surgical specimens and tyrosine phosphatase inhibitor treatment Samples of gastric cancers and corresponding normal mucosal tissues were obtained from specimens surgically resected from 27 patients at the National Cancer Center Hospital (Tokyo) in 1995. For western blotting and immunohistochemical staining to detect phosphotyrosine residues, fresh surgical specimens were treated with 1 m*M* sodium orthovanadate in phosphate-buffered saline containing 1 m*M* $CaCl_2$ under an atmospheric pressure of 1060 mmHg for 5 min in order to preserve phosphorylated tyrosine residues. One-half of each tissue sample was stored at −80°C until required, and the other was fixed overnight with absolute methanol at 4°C and embedded in paraffin.

Immunohistochemistry, western blotting and immunoprecipitation Tissue lysis, western blotting and immunoprecipitation were carried out using the methods described previously.[13] Mouse monoclonal antibodies against human E-cadherin (HECD-1; 10 mg/ml),[18] α- and β-catenin (2.5 mg/ml and 1 mg/ml; anti-α- and anti-β-catenin; respectively, Transduction Laboratories, Lexington, KY), phosphotyrosine residue (5 mg/ml; PY-20; Seikagaku Co., Tokyo) and EGF-R (500 ng/ml; mouse anti EGFr; ZYMED Laboratories Inc., San Francisco, CA) and rabbit anti-*c-erbB-2* gene product (500 ng/ml; anti-human *c-erbB-2* Gene Product; Nichirei, Tokyo), anti-human *c-met* gene product (5 mg/ml; Ab-*C-met* Gene Product/Hum; Seikagaku Co.) and anti-human K-*sam* gene product (1 mg/ml; KS1-2; kindly provided by Dr. M. Terada, Natl. Cancer Center Res. Inst., Tokyo[19]) antisera were used for immunohistochemical staining and western blotting analysis.

The immunohistochemical method using the avidin-biotin-peroxidase complex was previously described.[20] The reaction products were visualized with diaminobenzidine and the sections were counterstained with hematoxylin.

The immunohistochemical criteria for E-cadherin and catenin expression were as follows: when over 80%, 5–79% or less than 5% of the cancer cells were positively immunostained, the tumors were regarded as diffuse positive (diffuse), focal positive (focal) and loss of expression (loss), respectively. Cytoplasmic and nuclear localization of E-cadherin and catenins in more than 20% of the tumor cells were also recorded as C and N, respectively. Immunostaining of each of the four growth factor receptor-tyrosine kinases was considered positive when over 20% of the cancer cells were distinctively stained. Any carcinoma showing focal positive staining (less than 20% of the tumor cells) or very weak staining that was difficult to distinguish from the background level was regarded as negative (−). Phosphotyrosine immunoreactivity was classified as follows: when over 20% of the tumor cells showed positive cytoplasmic and membrane staining, the tumor was regarded as positive (+), but if such immunostaining was detected in less than 19% of the cancer cells or was absent, the tumor was regarded as negative (−).

Statistical analyses of immunostainings were made using the χ^2 test and differences between 2 populations were considered significant when confidence intervals were over 95% ($P<0.05$).

RESULTS

Preservation of phosphotyrosine residues in surgical specimens In order to ensure that sodium orthovanadate treatment preserved phosphotyrosine residues in the surgical specimens, western blotting and immunohistochemical staining with the anti-phosphotyrosine antibody, PY-20, of both fresh and stored normal gastric mucosae were performed. Sodium orthovanadate treatment preserved tyrosine phosphorylated residues in the gastric mucosa well even after frozen storage for 2 years (Fig. 1). Immunohistochemically, phosphotyrosine immunoreactivity was distributed at cell-cell and cell-matrix contact sites in nor-

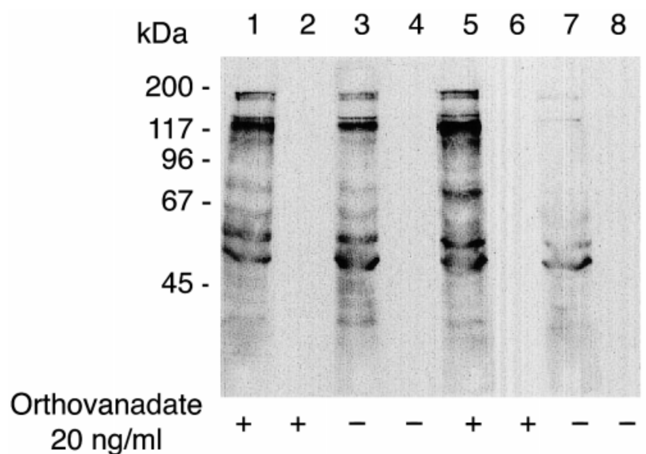

Fig. 1. Preservation of phosphotyrosine residues by sodium orthovanadate treatment. Western blot analysis using the anti-phosphotyrosine antibody PY-20 of normal gastric mucosa with (lanes 1, 2, 5 and 6) and without (lanes 3, 4, 7 and 8) sodium orthovanadate treatment. Tissue lysates of fresh resected materials (lanes 1, 2, 3 and 4) and materials stored frozen for 2 years (lanes 5, 6, 7 and 8) were electrophoresed and immunoblotted with PY-20 (lanes 1, 3, 5 and 7) and normal mouse IgG (lanes 2, 4, 6 and 8) as the primary antibodies.

mal gastric glands of mucosal tissues treated with sodium orthovanadate fixed in methanol and embedded in paraffin (Fig. 2A), but very weak or no immunoreactivity was observed in specimens processed without sodium orthovanadate treatment (Fig. 2B).

Immunohistochemical staining of gastric cancer specimens The expression of E-cadherin, catenins, the four receptor-type tyrosine kinases and phosphotyrosine residues is summarized in Table I (17 undifferentiated-type) and Table II (10 differentiated-type gastric cancers). Representative immunohistochemical stainings of E-cadherin, catenins, the growth factor receptors and phosphotyrosine residues in both undifferentiated- and differentiated-type gastric cancers are shown in Fig. 3.

In undifferentiated-type gastric cancers, loss of E-cadherin and α- and β-catenin expression was observed in 4, 8 and 3 of 17 tumors and focal positive expression in 7, 3 and 7, respectively. Diffuse cytoplasmic staining of E-cadherin (Fig. 3A), α-catenin and β-catenin (Fig. 3C) was observed in 4, 8 and 8, respectively. Cadherin and catenin were observed frequently in the cytoplasm of tumors showing diffuse positive E-cadherin and catenin immunostaining. Overexpression and nuclear accumulation of β-catenin were detected in one case (case 16 in undifferentiated-type gastric cancer) (Fig. 3I). *c-ErbB-2*, EGF-R, K-*sam* (Fig. 3E) and *c-met* were observed in 7, 13, 6 and 13 tumors, respectively. Expression of phosphotyrosine showed a cytoplasmic distribution in 8 (47%) tumors (Fig. 3G). The cytoplasmic distribution of phosphotyrosine residues correlated significantly with that of K-*sam* expression ($P<0.01$) and diffuse cytoplasmic staining of E-cadherin ($P<0.05$) and β-catenin ($P<0.05$).

In differentiated-type gastric cancers, all 10 tumors, with the exception of one cancer showing loss of β-catenin expression, expressed E-cadherin and α- and β-catenin. E-cadherin and α- and β-catenin in differentiated-type cancers showed membranous distributions similar to those in the corresponding normal gastric epithelia (Fig. 3, B and D). Expression of *c-erbB-2* (Fig. 3F), EGF-R and *c-met* was observed frequently in 8, 7 and 8 differentiated-type carcinomas, respectively, and K-*sam* expression was detected in one. Expression of phosphotyrosine (Fig. 3H) was detected at the cell membranes of 4 tumors and showed no correlation with growth factor receptor-tyrosine kinase expression. Overexpression and nuclear accumulation of β-catenin was detected in two tumors (case 4 and 9 in differentiated-type gastric cancer) (Fig. 3J).

Comparison of the immunohistochemical data for the two histological types showed that loss of E-cadherin ($P<0.05$) and β-catenin ($P<0.05$), and overexpression of K-*sam* ($P<0.05$) occurred significantly more frequently in undifferentiated-type carcinomas, whereas *c-erbB-2* expression was detected more frequently in the differentiated-type ($P<0.05$), but did not correlate with EGF-R or *c-met* expression.

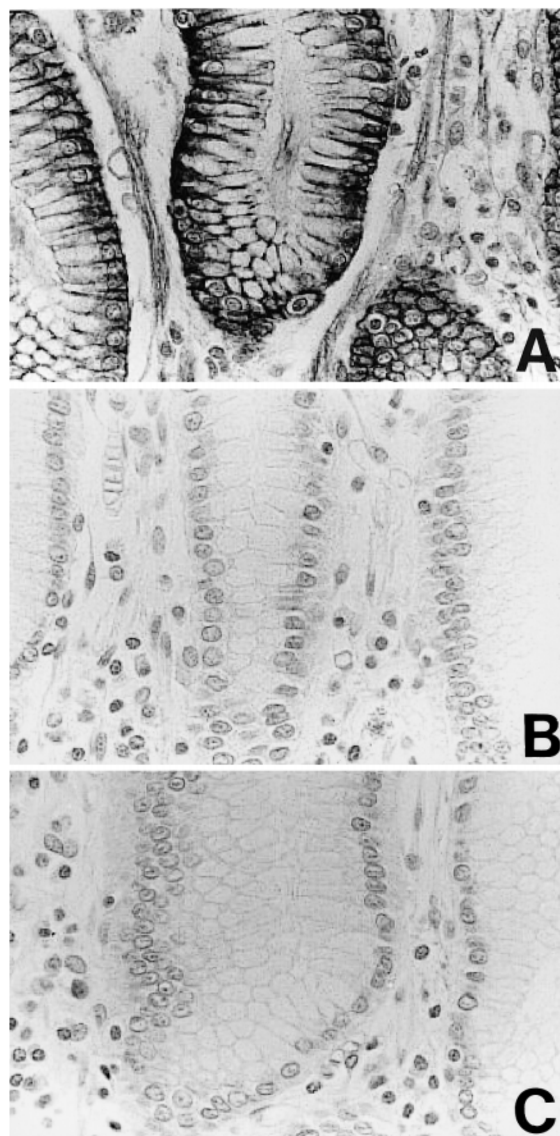

Fig. 2. Immunohistochemical stainings using PY-20 for methanol-fixed and paraffin-embedded sections of normal gastric mucosa treated with (A) or without (B) sodium orthovanadate. (C) Normal mouse IgG was applied for the serial section to (A) as the primary antibody for negative control staining. ×510.

Tyrosine phosphorylation of β-catenin In order to examine if β-catenin is tyrosine-phosphorylated in gastric cancer cells with loose cell-cell connections, five frozen stored samples of undifferentiated-type cancers showing cytoplasmic distribution of phosphotyrosine residues, E-cadherin and catenins were investigated. Tissue lysates were immunoprecipitated with the anti-β-catenin antibody,

Table I. Expression of E-Cadherin, Catenins, Growth Factor Receptors and Phosphotyrosine in Undifferentiated-type Gastric Cancers

Case No.	Histological type	E-Cadherin	α-Catenin	β-Catenin	*c-erbB-2*	EGF-R	K-*sam*	*c-met*	P-Tyr.
1	por2	diffuse	diffuse	diffuse	+	+	−	+	+
2	sig	diffuse (C)	diffuse (C)	diffuse (C)	+	+	+	+	+
3	por2	loss	loss	diffuse (C)	+	+	+	+	+
4	sig	diffuse (C)	diffuse (C)	diffuse (C)	−	−	+	+	+
5	muc	diffuse	diffuse (C)	diffuse (C)	+	+	+	+	+
6	por2	focal (C)	loss	focal (C)	−	+	−	−	+
7	sig	diffuse (C)	diffuse (C)	diffuse (C)	+	+	+	+	+
8	por2	diffuse	focal (C)	focal (C)	−	+	+	+	+
9	por2	focal	loss	focal	−	+	−	+	−
10	por2	loss	focal (C)	loss	−	+	−	+	−
11	por2	focal	loss	focal	−	+	−	−	−
12	por2	focal	loss	focal	−	−	−	−	−
13	por2	focal	focal (C)	focal	+	+	−	+	−
14	por1	focal	diffuse (C)	focal (C)	−	−	−	+	−
15	por2	focal	loss	loss	+	−	−	+	−
16	por2	loss	loss	diffuse (C, N)	−	+	−	−	−
17	por1	loss	loss	loss	−	+	−	+	−

EGF-R, epidermal growth factor receptor; P-Tyr., phosphotyrosine. Histological type is assigned according to the criteria of the Japanese Classification of Gastric Cancer by the Japanese Research Society for Gastric Cancer (1995).[33] por1, poorly differentiated adenocarcinoma (solid type); por2, poorly differentiated adenocarcinoma (non-solid type); sig, signet ring cell carcinoma; muc, mucinous adenocarcinoma. Immunohistochemical criteria were described in "Materials and Methods." Cytoplasmic and nuclear localizations of E-cadherin and catenins were recorded as C and N, respectively.

Table II. Expression of E-Cadherin, Catenins, Growth Factor Receptors and Phosphotyrosine in Differentiated-type Gastric Cancers

Case No.	Histological type	E-Cadherin	α-Catenin	β-Catenin	*c-erbB-2*	EGF-R	K-*sam*	*c-met*	P-Tyr.
1	tub2	diffuse	diffuse	diffuse	+	−	−	+	+
2	tub2	diffuse	diffuse	diffuse	+	+	−	+	+
3	tub2	diffuse	diffuse	diffuse	+	+	+	+	+
4	pap	diffuse	diffuse	diffuse (C, N)	+	+	−	+	+
5	tub2	diffuse	diffuse	diffuse	+	−	−	−	−
6	tub2	diffuse	diffuse	diffuse	+	+	−	+	−
7	tub2	diffuse	diffuse	diffuse	−	−	−	−	−
8	tub1	diffuse	diffuse	diffuse	+	+	−	+	−
9	pap	diffuse	diffuse	diffuse (C, N)	−	+	−	+	−
10	tub2	diffuse	diffuse	loss	+	+	−	+	−

EGF-R, epidermal growth factor receptor; P-Tyr., phosphotyrosine. Histological type is assigned according to the criteria of the Japanese Classification of Gastric Cancer by the Japanese Research Society for Gastric Cancer (1995).[33] tub1, well differentiated tubular adenocarcinoma; tub2, moderately differentiated tubular adenocarcinoma; pap, papillary adenocarcinoma. Immunohistochemical criteria were described in "Materials and Methods." Cytoplasmic and nuclear localizations of E-cadherin and catenins were recorded as C and N, respectively.

then blotted onto a polyvinylidene difluoride membrane and tyrosine phosphorylation was detected by immunostaining with the anti-phosphotyrosine antibody PY-20. Tyrosine-phosphorylated β-catenin was clearly detected in one adenocarcinoma (case 2). The β-catenin expression levels of both the normal mucosa and cancer tissue of this case were virtually identical (Fig. 4A), but tyrosine-phosphorylated β-catenin was detected only in the cancer tissue (Fig. 4B). When cell lysates were immunoprecipitated with the anti-phosphotyrosine antibody, β-catenin was detected in the immunoprecipitates of the cancer tissue, but not in those of the normal mucosa (Fig. 4C).

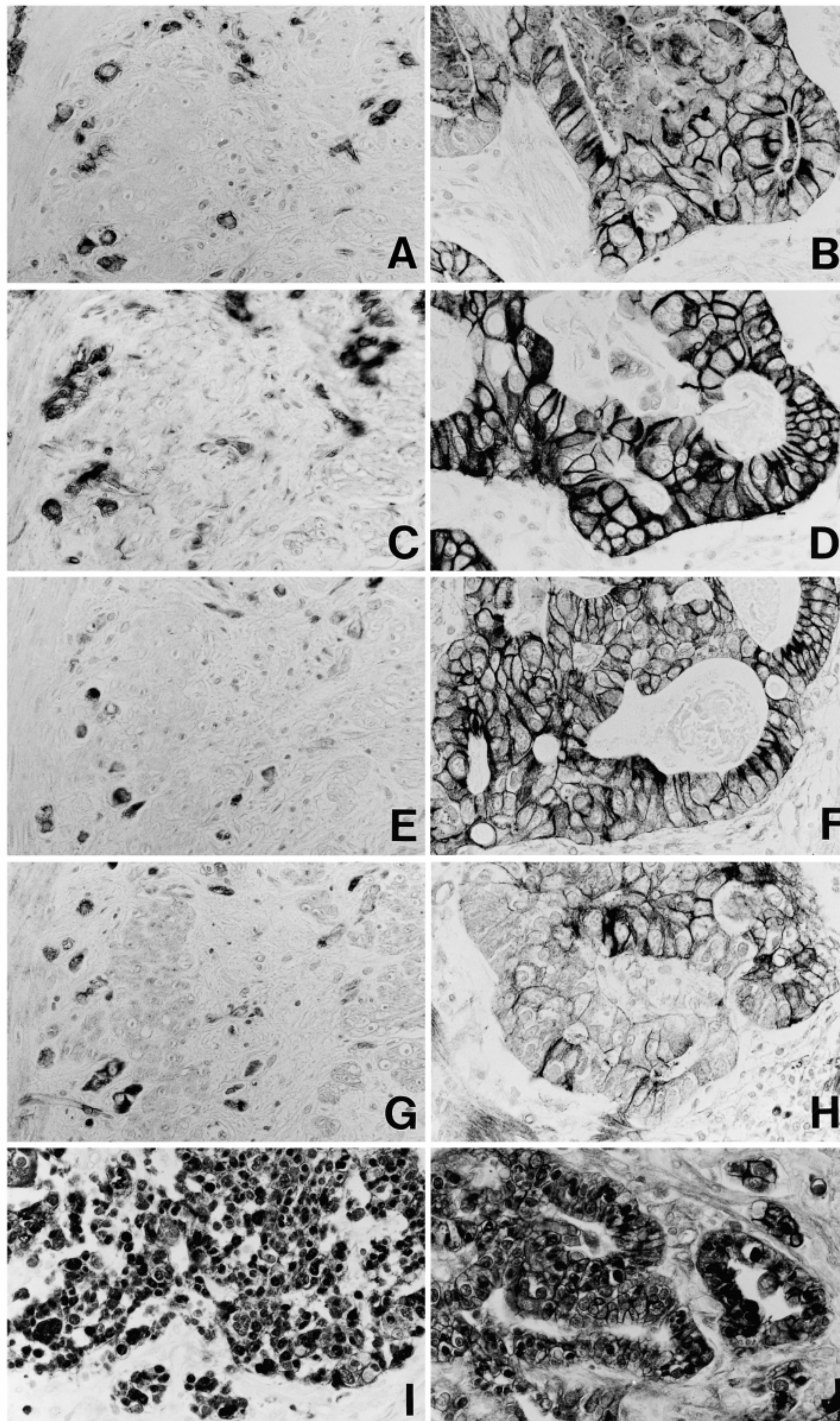

Fig. 3. Representative immunohistochemical stainings of E-cadherin (A and B), β-catenin (C and D), K-*sam* (E), *c-erbB-2* (F) and phosphotyrosine residues (G and H) in surgical specimens of undifferentiated-type (A, C, E and G; case 2) and differentiated-type (B, D, F and H; case 1) gastric cancers. ×410. Diffuse nuclear accumulation of β-catenin in surgical specimens of undifferentiated-type (I; case 16) and differentiated-type (J; case 4) adenocarcinomas. ×410.

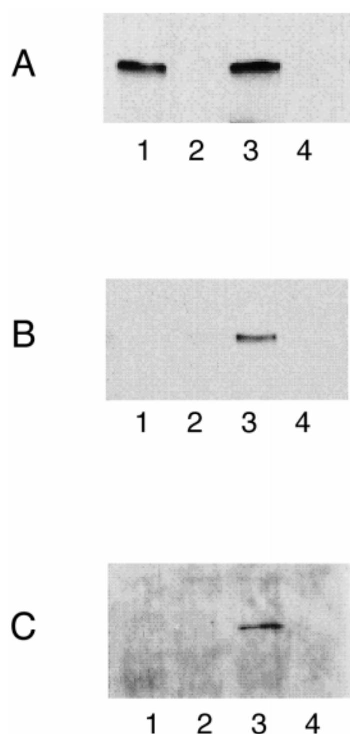

Fig. 4. Tyrosine phosphorylation of β-catenin in a surgically resected specimen of a poorly differentiated adenocarcinoma (case 2). Detection of β-catenin (A) and phosphotyrosine residues (B) in co-immunoprecipitates using the anti-β-catenin antibody. Lanes 1 and 2: normal mucosa; 3 and 4: cancerous tissue; 1 and 3: immunoprecipitated with the anti-β-catenin antibody and 2 and 4: immunoprecipitated with normal mouse IgG. (C) Detection of β-catenin in immunoprecipitates using the PY-20 antibody. Lanes 1 and 2: normal mucosa; 3 and 4: cancerous tissue; 1 and 3: immunoprecipitated with the anti-β-catenin antibody and 2 and 4: immunoprecipitated with normal mouse IgG.

DISCUSSION

The finding that undifferentiated-type gastric cancers frequently showed loss or reduced expression of E-cadherin and α- and β-catenin was consistent with that of our previous immunohistochemical study.[21] Reduced expression in undifferentiated-type gastric cancers may be attributable to mutation of E-cadherin-catenin molecules or methylation of the promoter region of the E-cadherin gene, as reported previously.[9,10] In fact, Becker and Hoeler[16] reported that 42% of surgical specimens of diffuse-type gastric cancers, which correspond to undifferentiated-type gastric cancers, possessed E-cadherin gene mutations. In addition to the loss or reduced expression of E-cadherin and α- and β-catenin in undifferentiated-type cancers, diffuse cytoplasmic expression of E-cadherin and α- and β-catenin was observed frequently in eight of 17 undifferentiated-type cancers. Such diffuse cytoplasmic staining of these molecules, demonstrated in immunohistochemical studies, was suggested to reflect dysfunction of the cadherin cell adhesion system.[20–24] In this study, diffuse cytoplasmic staining of E-cadherin and β-catenin correlated significantly with the cytoplasmic distribution of phosphotyrosine residues. Taken together with previous findings that activation of tyrosine kinase receptors resulted in dysfunctional cadherin-mediated cell adhesion,[11,12] our results suggest that alteration of the tyrosine phosphorylation status of gastric cancer cells may cause dysfunction of cadherin cell adhesion molecules.

In addition to its association with cytoplasmic cadherin-catenin molecules, we found that the cytoplasmic phosphotyrosine distribution pattern correlated significantly with that of K-*sam* oncogene protein expression. The K-*sam* oncogene, first detected as a highly amplified gene in a stomach cancer cell line, KATO-III,[25] has been proven to be a receptor for keratinocyte growth factor.[26] Overexpression of K-*sam* occurs specifically in undifferentiated-type gastric cancers and is associated with a poor prognosis.[19] We observed a physical association between β-catenin and the K-*sam* oncogene product in some human poorly differentiated adenocarcinoma cell lines (unpublished data). These data led us to hypothesize that dysfunction of the E-cadherin-mediated cell adhesion system in undifferentiated-type gastric carcinomas may result from β-catenin phosphorylation through activation of the K-*sam* gene product. However, we could detect tyrosine-phosphorylated β-catenin in only one surgical specimen of an undifferentiated-type adenocarcinoma in this study. The tumor cells of undifferentiated-type adenocarcinomas are surrounded by rich stroma, which may contaminate samples. Further studies using microdissection methods, which can isolate single cancer cells from the surrounding rich stromal tissues, are needed to help elucidate the mechanisms responsible for dysfunctional cell-to-cell adhesion in undifferentiated-type gastric cancers.

The protein coded by *APC* tumor suppressor gene was demonstrated to regulate β-catenin degradation[27] and deletion or truncation of APC protein caused by gene mutation inhibits β-catenin degradation, resulting in accumulation of β-catenin in both the cytoplasm and nucleus. *APC* gene mutations have been detected in gastric cancers mainly in differentiated-type adenocarcinomas, but they were also observed in some signet ring cell carcinomas.[28] The diffuse nuclear accumulation of β-catenin we observed in three tumors, two differentiated and one undifferentiated, may have been due to alteration of the APC-β-catenin system, as observed in familial adenomatous polyposis patients.[29] More recently, mutations of the glycogen synthase kinase 3-β recognition site at the N-terminal portion of β-catenin were reported to occur frequently in cancer cell lines showing no alterations of the

APC gene and these mutations stabilized β-catenin.[30, 31] The stabilized β-catenin binds to Tcf4/Lef1 DNA transcription factors and transfers proliferating signals in normal and cancer cells.[32] In this study, we observed nuclear accumulation of β-catenin in some cancer cells with diffuse cytoplasmic distribution of β-catenin in seven of eight undifferentiated-type cancers which showed cytoplasmic localization of phosphotyrosine residues. It is not clear whether cytoplasmic distributed β-catenin observed in undifferentiated-type gastric cancers affects not only the cadherin-mediated cell adhesion, but also gastric carcinogenesis and cancer cell progression through activation of β-catenin Tcf4/Lef1-mediated transcription. Further studies to elucidate the correlations between activation of receptor-type tyrosine kinases and diffuse cytoplasmic distribution of β-catenin are under way in our laboratory.

ACKNOWLEDGMENTS

This study was supported partly by a Grant-in-Aid from the Ministry of Health and Welfare, partly by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture and partly by Special Coordination Funds of the Science and Technology Agency of Japan.

(Received April 13, 1998/Revised June 11, 1998/Accepted June 15, 1998)

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