tively), plasminogen activator inhibitors (PAI-1, PAI-2), and uPA receptors, have been associated with tumor aggressiveness in a variety of solid malignant tumors 8,9].

Several reports recently showed that the regulation of MMPs is mediated by the cytokines interleukin-1 10–12], transforming growth factor-β 13], and tumor necrosis factor-α 11] in stromal cells of the endometrium. Some studies indicated that stromal fibroblasts assisted with the invasion of tumor cells through the extracellular matrix by producing elevated amounts of proteolytic enzymes after interaction with soluble factors (e.g., βFGF) 14–16]. A three-dimensional (3-D) matrix composed of reconstituted collagen fibers can create culture conditions more similar to in vivo tissues than routine dish cultures in vitro 17] and has been used to evaluate the remodeling process 18,19] and tumor invasion 20–22]. However, it is sometimes difficult to use collagen gel, in which tumor and stromal cells are cocultured, for immunohistochemistry, because it is easily broken during the process of fixation and embedding in paraffin, especially in the case of long-term culture 23].

Previously we found by invasion assay that under conditions with 17β-estradiol the invasiveness of Ishikawa cells was enhanced according to the expression of MMP-1, -7, and -9 and Ets-1, suggesting that activation of ER-α by estrogen resulted in tumor progression by stimulating cell growth and invasiveness via acceleration of the expression of MMPs 24]. In another study, we demonstrated the expression and localization of MMP-1, -2, -3, -7, and -9 and TIMP-1 in endometriosis tissues by immunohistochemistry and it appeared that the destruction of the surrounding matrix by the endometriosis might be caused by various MMPs, which are mainly produced in stromal cells 25]. In another previous study, we demonstrated that in vitro invasive abilities of endometrial carcinoma cell lines depend on the degree of cell differentiation and the origin of cell lines. A poorly differentiated carcinoma cell line (NUE-1) and a cell line derived from metastatic lymph node (SNG-M) were more invasive than moderately (HEC-1A, HEC-1BE) and well-differentiated (HEC-6, Ishikawa) cell lines 26]. To examine the interaction between endometrial cancer cells and fibroblasts for tumor invasion, in this study we carried out 3-D coculture of endometrial cancer cells and fibroblasts in human placenta derived collagen sponges and analyzed the expression and localization of MMPs and PAs in these cells by immunohistochemistry.

Materials and methods

Extraction of collagen

Collagen was prepared from placenta as described by Niyibizi et al. 27]. The amnion and chorion were dissected away from human placentas and the placentas were suspended in cold distilled water at 4°C for 3 days with two changes of water per day. The placentas were then ground in a meat grinder and suspended in cold distilled water at 4°C for 2 days with two changes of water per day. Each change was accomplished by filtering and squeezing the ground placenta through cheesecloth. The ground placentas were then suspended in 0.5 M sodium acetate at 4°C for 16 h; filtered, and squeezed through cheesecloth and washed extensively with cold distilled water. The placentas were then weighed and suspended in 0.5 M acetic acid (300 g/liter, wet wt). Pepsin (Sigma Chemical, St. Louis, MO) was added (300 mg/liter) and the tissue was digested for 16 h at 4°C. The insoluble residue was pelleted by centrifugation (2500g for 1 h). NaCl (1.0 M) was added to the supernatant and types I, III, IV, and V collagen were collected by centrifugation at 2500g for 30 min.

Collagen sponge

This procedure was performed by modifying that of Berthod et al. 28]. First, 2.25 g of the collagen hydrated in 150 mL of distilled water with 0.1% acetic acid was mixed with 0.625 g of chitosan (85% deacetylated, Sigma Chemical) dissolved in 75 mL of water and 0.25 g of GAG (chondroitin sulfate, Sigma Chemical) in 25 mL of distilled water. After 1 mL/well (1.8 cm^2) of the solution was poured into 24-well plates (Falcon, Becton–Dickinson Labware, Franklin Lakes, NJ) and frozen at 80°C for 1 h, 100% ethanol, 0.5 mL/well, was poured into each well for dehydration. After these sponges, were slowly melted at room temperature, they were immersed in a solution of 2.5% glutaraldehyde, rinsed with Hanks' balanced salt solution (Gibco/BRL Life Technologies, Gaithersburg, MD) thoroughly, and then incubated in minimal essential medium (MEM) (Gibco/BRL Life Technologies) with 10% FCS overnight.

3D coculture

As summarized in Fig. 1. fibroblasts (five passages) derived from the human uterine cervix were trypsinized and 100 μl of suspension of 5×10^6 cells/mL was added on top of the sponges and incubated at 37°C and 5% CO_2 for 14 days with a change of MEM every 3 days. Then 5×10^5 endometrial cancer cells/100 μl of medium (Ishikawa, HEC-1A, HEC-1BE, HEC-50B, HEC-108, KLE, RL-952, SNG-M, or SNG-2) were added on top of the collagen sponges, in which fibroblasts were growing, and incubated for 14 days in MEM with 10% FCS. The products of 3-D coculture were fixed overnight in 10% buffered formalin, dehydrated, and embedded in paraffin. Sections (5 μm) were stained with hematoxylin–eosin and these serial sections were used for immunohistochemistry. The numbers per 10 high-power fields (HPF) of cancer nests that had invaded the layer of extracellular matrix were counted.

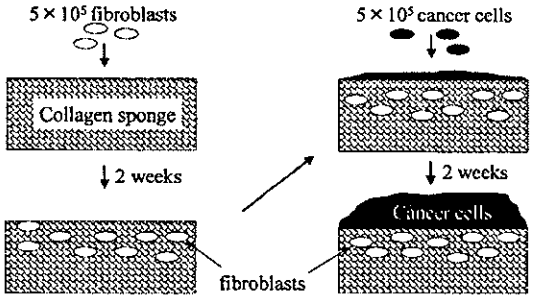

Fig. 1. Diagram of 3-D coculture of endometrial cancer cells and fibroblasts on collagen sponges. Fibroblasts derived from human uterine cervix were trypsinized and 100 μl of a suspension of 5×10^8 cells/mL was added on top of their sponges and incubated at 37°C and 5% CO_2 for 14 days with a change of MEM every 3 days. Then 5×10^5 endometrial cancer cells/100 μl of medium (Ishikawa, HEC-IA, HEC-1BE, HEC-50B, HEC-108, KLE, RL-952, SNG-M, or SNG-2) was added on the top of collagen sponges in which fibroblasts were growing and incubated for 14 days in MEM with 10% FCS.

Immunochemistry

Paraffin-embedded sponges were cut, floated onto albumin-coated slides, dried at 56°C, deparaffinized in xylene, rehydrated, and washed with phosphate-buffered saline (PBS) for 15 min at room temperature. Specimens were treated in a microwave oven in 0.01 mol/L citrate buffer (pH 6.0) for 30 min at 100°C, slowly cooled to room temperature, and then washed with PBS for 5 min at room temperature. After endogenous peroxidase was quenched with 3% hydrogen peroxide in PBS for 10 min at room temperature, the sections were blocked in 10% rabbit serum for 60 min at room temperature and incubated for 16 h at 4°C in anti-MMP-1, -2, -7, or -9, anti-MT1-MMP, TIMP-1, and -2 (Fuji Chemical Ltd., Toyama, Japan) diluted 1:200 and tPA and uPA (Santa Cruz Biotechnology, CA) diluted 1:100 in PBS. After two washes with PBS, they were incubated for 1 h at room temperature with a biotin anti-mouse antibody. They were then washed and incubated for 1 h with peroxidase streptavidin (Nichirei). The color reaction was developed by the silver intensification procedure described previously 29]. Staining evaluation was performed by two independent observers (R.T. and T.S.) without knowledge of clinical outcome. For each tissue sample, the intensity of immunostaining was graded negative, weak, or intensive for both epithelial and stromal cells.

Electron microscopy

For electron microscopy, the collagen sponges were fixed in 2.5% (v/v) glutaraldehyde with 0.1 M phosphate buffer (pH 7.4). They were then cut into approximately 1-mm^3 pieces, postfixed in 1% osmium tetoxide after dehydration, and embedded in Epon 812 and ultrathin sections were then prepared. The specimens were examined under a JEOL 1200 EX electron microscope after uranyl acetate and lead citrate staining.

Results

3-D coculture and count of invading cancer nests

As shown in Fig. 2a, fibroblasts seeded in the sponge migrated into the alveolar network and formed a layer of extracellular matrix on the top of the sponge after 14 days of culture. Then 14 days after endometrial cancer cells were seeded on the top of the collagen sponge in which fibroblasts were growing, the endometrial cancer cells composed stratiform or glandular structures on the layer of extracellular matrix and divided into epithelial elements and stromal elements. Some cells showed invading cancer nests breaking the layer of extracellular matrix, and we counted the numbers of cancer nests (Fig. 3). As shown in Fig. 2b, Ishikawa, KLE, RL-952, SNG-M, and SNG-2 cells showed weak invasiveness. The number of cancer nests invading through the layer of extracellular matrix in 10 HPF was 0.7 ± 0.1 (SE) in Ishikawa, 1.4 ± 0.2 in KLE, 1.7 ± 0.2 in RL-952, 3.1 ± 0.3 in SNG-M, and 3.6 ± 0.3 in SNG-2. On the other hand, HEC-1A (Fig. 2c), HEC-1BE (Fig. 2d), HEC-108, and HEC-50B showed higher invasiveness. The numbers of cancer nests invading through the layer of extracellular matrix in 10 HPF was 8.9 ± 0.6 in HEC-1A, 11.5 ± 0.8 in HEC-1BE, 19.6 ± 1.6 in HEC-108, and 19.8 ± 1.9 in HEC-50B.

Transmission electron microscopy

Ishikawa cells, which rarely invaded the layer of extracellular matrix, and HEC-1A, which had 8.9 ± 0.6/HPF invading cancer nests, were observed by transmission electron microscopy (Fig. 4). Ishikawa cells grew on the layer of extracellular matrix and formed a glandular structure. The layer of extracellular matrix was well preserved, the extracellular matrix was observed around fibroblasts, and the bottom layer of cancer cells was linear in the collagen sponges in which fibroblasts and Ishikawa were cocultured. On the other hand, the layer of extracellular matrix was unclear, the extracellular matrix around fibroblasts was degraded, and the bottom layer of cancer cells was irregular in the collagen sponges in which fibroblasts and HEC-1A were cocultured. Cell adhesion of HEC-1A was weak and the glandular formation was not observed.

Immunohistochemical localization of MMPs and PAs

To examine the interaction between cancer cells and fibroblasts in the collagen sponges, the expression and localization of MMP-1, -2, -7, and -9, MT1-MMP, TIMP-1 and -2, tPA, and uPA were immunohistochemically analyzed. In this study, we used the cell lines Ishikawa, which was rarely invasive, and HEC-1A and HEC-1BE, which were highly invasive. The results are summarized in Table 1. In Ishikawa and cocultured fibroblasts, all proteins analyzed in this study were posi-

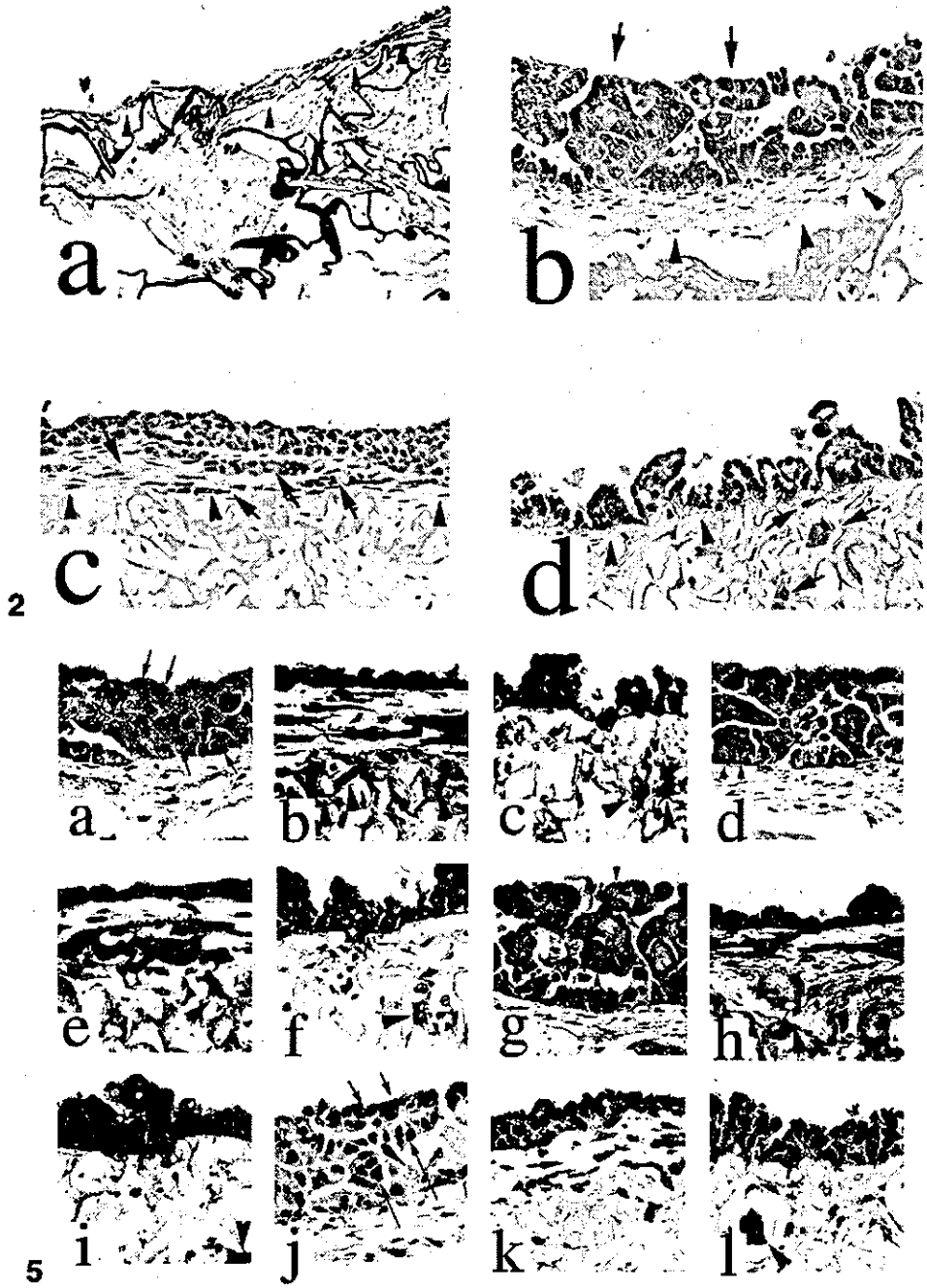

Fig. 2. Layer of extracellular matrix and 3-D structure of cancer cells growing on collagen sponges. Fibroblasts seeded in the sponge migrated into the alveolar network and formed a layer of extra cellular matrix on the top of the sponge after 14 days of culture (a, arrowhead). 14 days after endometrial cancer cells were seeded on the top of the collagen sponge in which fibroblasts were growing, endometrial cancer cells composed stratiform or glandular structures (b, arrow) on the layer of extracellular matrix (b, arrowhead), which divided into epithelial elements and stromal elements. Some cell lines had invading cancer nests (c and d, arrow) breaking the layer of extra cellular matrix (c and d, arrowhead), and we counted the numbers of cancer nests. Ishikawa (b) had weak invasiveness. On the other hand, HEC-1A (c) and HEC-1BE (d) had higher invasiveness. Original magnification ×400.

Fig. 5. Immunohistochemistry of MMPs and PAs in endometrial cancer cells and fibroblasts cultured on collagen sponges. MMP-1 (a) and tPA (j) were localized only around the nucleus (arrow) and MMP-7 (d) and -9 (g) were localized only in the cytoplasm (arrowhead) in Ishikawa, but detected in all parts of cells in HEC-1A (b, e, h, k) and HEC-1BE (c, f, i, l). In HEC-1A, MMP-1 (b), -7 (e), and -9 (h) and uPA were intensively stained in both cancer cells and fibroblasts (large arrowhead). HEC-1BE and cocultured fibroblasts (large arrowhead) had expression patterns similar to those of HEC-1BE. a, d, g and i: Ishikawa, b, e, h and k: HEC-1A, c, f, i, l: HEC-1BE. a, b, and c: MMP-1, d, e, and f: MMP-7, g, h, and i: MMP-9, j, k, and l: uPA. Arrowhead: invading cancer cells. Original magnification, ×400.

tively detected. MMP-1 was localized only around the nucleus in Ishikawa cells (Fig. 5a), but detected in whole cells in HEC-1A (Fig. 5b) and HEC-1BE (Fig. 5c). Compared to Ishikawa (Fig. 5a, 5d, 5g, 5j), HEC-1A and HEC-1BE and cocultured fibroblasts showed strong expression in some proteins. In HEC-1A, MMP-1 (Fig. 5b),

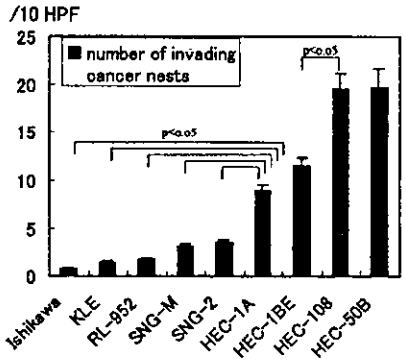

Fig. 3. Number of cancer nests invading the layer of extracellular matrix. The number of cancer nests invading the layer of extracellular matrix in 10 HPF was 0.7 ± 0.1 (SE) in Ishikawa, 1.4 ± 0.2 in KLE, 1.7 ± 0.2 in RL-952, 3.1 ± 0.3 in SNG-M, and 3.6 ± 0.3 in SNG-2. On the other hand, HEC-1A, HEC-1BE, HEC-108, and HEC-50B showed higher invasiveness. The number of cancer nests invading through the layer of extra cellular matrix in 10 HPF was 8.9 ± 0.6 in HEC-1A, 11.5 ± 0.8 in HEC-1BE, 19.6 ± 1.6 in HEC-108, and 19.8 ± 1.9 in HEC-50B.

-7 (Fig. 5e), and -9 (Fig. 5h), MT1-MMP, TIMP-2, and uPA (Fig. 5k) showed intensive staining in both cancer cells and fibroblasts, but MMP-2 and tPA were weakly expressed in both cell lines. HEC-1BE and cocultured fibroblasts showed expression patterns similar to those of HEC-1A (Fig. 5c, f, i, and l), but TIMP-1 was weakly detected in the cocultured fibroblasts. MMP-2 and tPA were weakly detected both in cancer cells and in fibroblasts for all three cell lines.

Discussion

In this study, we performed 3-D coculture of endometrial cancer cells and fibroblasts in human placenta derived collagen sponges and analyzed expression and localization of MMPs, TIMPs, uPA, and tPA in these cells by immunohistochemistry to investigate the interaction between endometrial cancer cells and fibroblasts in tumor invasion.

Collagen gel constitutes a valuable tool for the study of cell–matrix interactions 30–33], but the low collagen synthesis does not promote the construction by closed fibroblasts of a newly synthesized matrix made of various human proteins, which should be the best way to produce a highly differentiated environment around cells 23]. Berthod et al. demonstrated that synthesis of collagen and protein was markedly stimulated inside a collagen sponge culture system compared with a collagen gel (6- and 2.4-fold increase, respectively) 28]. In our study, fibroblasts migrated into the alveolar network and formed a layer of extracellular matrix on the top of the sponge after 14 days of culture. Furthermore, 14 days after endometrial cancer cells were seeded on the top of the collagen sponge in which fibroblasts were growing, endometrial cancer cells composed stratiform or glandular structures on the layer of extracellular matrix, which divided into epithelial elements and stromal elements. Thus, our 3-D culture system using human placenta derived collagen was useful as it could be employed for long-term culture and it was possible to observe the interaction between cancer cells and stromal cells histologically.

Some cell lines formed invading cancer nests breaking the layer of extracellular matrix, and we counted the number of cancer nests. In this study, Ishikawa, KLE, RL-952, SNG-M, and SNG-2 cells had weak invasiveness. On the other hand, HEC-1A, HEC-1BE, HEC-108, and HEC-50B cells had higher invasiveness. Electron microscopic examination demonstrated that the layer of extracellular matrix was well preserved, the extracellular matrix was observed around fibroblasts, and the bottom layer of cancer cells was linear in the collagen sponges in which fibroblasts and Ishikawa cells were cocultured. On the other hand, the extracellular matrix around fibroblasts was degraded and the bottom layer of cancer cells was irregular in the collagen sponges in which fibroblasts and HEC-1A cells were cocultured. These results suggested that cancer cells invaded the stroma, destroying the extracellular matrix existing around fibroblasts.

Previously we demonstrated, by invasion assay, under conditions with 17β-estradiol, that the invasiveness of Ishikawa cells was enhanced according to the expression of MMP-1, -7, and -9 and Ets-1, suggesting that activation of ER-α by estrogen resulted in tumor progression by stimulating cell growth and invasiveness via acceleration of the expression of MMPs 24]. In another study, we evaluated the expression and localization of MMP-1, -2, -3, -7, and -9 and TIMP-1 in endometriosis tissues by immunohistochemistry and it was suggested that the destruction of the surrounding matrix by the endometriosis might be caused by various MMPs, which were mainly produced in stromal cells 25]. The plasminogen activating system also plays a key role in the cascade of tumor-associated proteolysis leading to extracellular matrix degradation and stromal invasion. Changes in the expression of this system, consisting of urokinase- and tissue-type plasminogen activators (uPA and tPA, respectively), plasminogen activator inhibitors (PAI-1, PAI-2), and the uPA receptor, have been associated with tumor aggressiveness in a variety of solid malignant tumors 8,9]. In this study, compared to Ishikawa, HEC-1A and HEC-1BE and cocultured fibroblasts showed strong expression of some proteins. MMP-1, -7, and -9, MT1-MMP, and uPA were intensively stained in both cancer cells and fibroblasts. This enhanced expression of MMPs and uPA might result in the high invasive capacity of HEC-1A and HEC-1BE.

There are several lines of evidence indicating that fibroblasts adjacent to cancer cells produce and release a number of soluble paracrine factors such as FGF(s) 34], transforming growth factor-β 35], IGFs 36], and hepatocyte growth factor 37] and/or the ability to respond to epithelial-derived signals 38]. Several reports have recently shown that the regulation of MMPs is mediated by the cytokines interleukin-1 10–12], transforming growth factor-β 13], and tumor necrosis factor-α

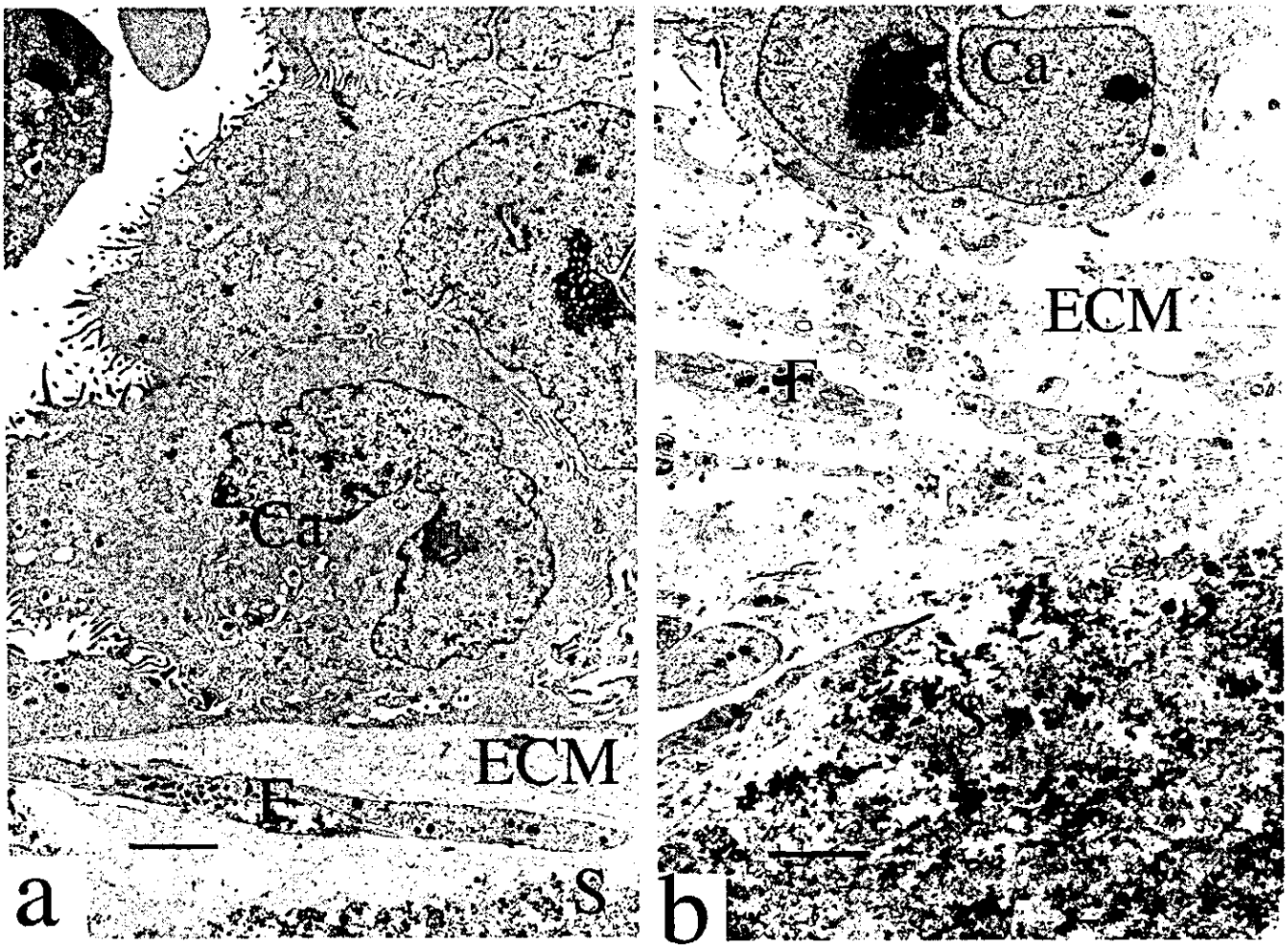

Fig. 4. Electron microscopy of endometrial cancer cells and fibroblasts cultured on collagen sponges. Ishikawa, which rarely invaded in the basement like structure, and HEC-1A, which had invading cancer nests 8.9 ± 0.6/HPF, were observed by transmission electron microscopy. (a) The layer of extracellular matrix was well preserved, the extracellular matrix was observed around fibroblasts, and the bottom layer of cancer cells was linear in the collagen sponges, in which fibroblasts and Ishikawa were cocultured. (b) On the other hand, the layer of extracellular matrix was unclear, the extracellular matrix around fibroblasts was degraded, and the lower bottom of cancer cells was irregular in the collagen sponges in which fibroblasts and HEC-1A were cocultured. S, collagen sponge; F, fibroblast; Ca, cancer cell; ECM, extracellular matrix. Bar, 2 μm.

11] in stromal cells of the endometrium. Expression of uPA is also stimulated by EGF in endometrial stromal cells 39]. From the evidence, it was suggested that expression of MMPs and uPA activated locally secreted paracrine factors in fibroblasts adjacent to HEC-1A and HEC-1BE.

We believe that our 3-D culture system has merits because the interaction between cancer cell and stromal cells can be visually analyzed by immunohistochemistry and experiments lasting for a long period, at least 2 weeks, are possible. Furthermore, it is expected that some animal, e.g., nude mice, experiments can be replaced by the experiments using this culture system.

Table 1
Immunohistochemistry of MMPs and PAs in endometrial cancers and fibroblasts

Cell line		MMP1	MMP2	MMP7	MMP9	MT1MMP	TIMP1	TIMP2	uPA	tPA
IK	Carcinoma	1+	1+	1+	1+	1+	1+	2+	1+	1+
	fibroblast	1+	1+	1+	1+	1+	1+	2+	1+	1+
HEC1A	Carcinoma	2+	1+	2+	2+	2+	2+	2+	2+	1+
	fibroblast	2+	1+	2+	2+	2+	2+	2+	2+	1+
HEC1BE	Carcinoma	2+	1+	2+	2+	2+	2+	2+	2+	1+
	fibroblast	2+	1+	2+	2+	2+	1+	2+	2+	1+

Acknowledgments

HEC-1A, HEC-1BE, HEC-50B, and HEC-108 were kindly given by Dr. Kuramoto (Kisasato University, Japan), SNG-II and SNG-M were from Dr. Nozawa (Keio University, Japan). Ishikawa was from Dr. Nishida (Tsukuba University, Japan), SPAC-IL was from Dr. Hirai (Cancer Institute Hospital, Japan), and KLE and RL-952 were provided by the ATCC.

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Hypermethylation in Promoter Region of E-cadherin Gene Is Associated with Tumor Dedifferention and Myometrial Invasion in Endometrial Carcinoma

Tsuyoshi Saito, M.D., Ph.D.[1]
Makoto Nishimura, M.D.[1]
Hiroshi Yamasaki, Ph.D.[2]
Ryuichi Kudo, M.D., Ph.D.[1]

[1] Department of Obstetrics and Gynecology, Sapporo Medical University School of Medicine, Sapporo, Japan.

[2] School of Science and Technology, Kwansei Gakuin University, Sanda, Japan.

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Address for reprints: Tsuyoshi Saito, M.D., Ph.D., Department of Obstetrics and Gynecology, Sapporo Medical University, S-1, W-16, Chuo-ku, Sapporo 060-0061, Japan; Fax: 011 (+81)-11-614-0860; E-mail: tsaito@sapmed.ac.jp

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BACKGROUND. Loss of E-cadherin expression is associated with aberrant 5′ CpG island methylation in various tumors.

METODS. The authors analyzed the methylation status and immunohistochemical expression of E-cadherin in 142 endometrial tissues, consisting of 21 normal endometria, 17 endometrial hyperplasias, and 104 endometrial carcinomas.

RESULTS. All normal endometria and endometrial hyperplasias showed positive staining of E-cadherin, and methylation of the E-cadherin gene was not detected in any samples. In endometrial carcinoma, the positive ratio of methylation was higher and was associated with tumor dedifferention and myometrial invasion. In G1 endometrial adenocarcinomas, 66.7% showed positive staining and 33.3% showed heterogeneous staining. Methylation of the E-cadherin gene was detected in 15.6%. In G2 tumors, 19.0% showed positive staining, 69.0% showed heterogeneous staining and 11.9% showed negative staining. Methylation of the E-cadherin gene was found in 50.0%. In G3 tumors, 9.1% showed positive staining, 54.5% showed heterogeneous staining and 36.3% showed negative staining. Methylation of the E-cadherin gene was found in 81.8% of the tumors. Of the samples with no-myometrial invasion, 23.1% had methylation. In those with invasion in less than half of the myometrium, 28.6% did and in those with invasion of half or more of the myometrium, 55.6% had methylation. Of samples that did not have lymph node metastasis, 33.7% had methylation, whereas of samples that had lymph node metastasis, 60.0% had methylation.

CONCLUSIONS. This is the first report to analyze methylation of the E-cadherin gene promoter of endometrial carcinoma and the evidence suggests that methylation of the E-cadherin gene occurs in association with the acquisition of invasive capacity.
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KEYWORDS: endometrial carcinoma, E-cadherin, beta-catenin, cell adhesion, carcinogenesis, invasion, metastasis, intercellular communication, connexin.

Cadherins are a family of cell-cell adhesion molecules essential for tight connection between cells.[1] E-cadherin is the major cadherin molecule expressed in epithelial cells. The cadherin-mediated cell adhesion system is known to act as an invasion suppressor system in cancer cells, since noninvasive cells can be transformed into invasive ones when treated with antibodies to block cadherin's function or with cadherin-specific antisense RNA;[2,3] transfection of human cancer cell lines with E-cadherin cDNA can reduce their invasiveness.[4] In fact, immunohistochemical examination has revealed that decreased E-cadherin expression is associated with tumor dedifferentiation and progression in endometrial carcinoma[5] and many other tumors.[6–10]

DNA methylation in the promoter regions of many genes is associated with the regulation of gene expression. It results in transcriptional

silencing of the gene, either through a direct effect or via a change in the chromatin conformation that inhibits transcription.[11] The transformation of normal mammary epithelial cells into carcinoma and the subsequent progression to invasion and metastasis involve the accumulation of numerous genetic hits, including the activation or amplification of dominant oncogenes and the deletion or inactivating mutation of key tumor suppressor genes.[12] It has recently become evident that tumor suppressor genes may also be transcriptionally silenced in association with aberrant promoter-region CpG island methylation.[13,14] Loss of E-cadherin expression is also associated with aberrant 5′ CpG island methylation in various tumors.[15–17] In endometrial carcinoma, aberrant promoter-region CpG island methylation has been reported in some genes such as estrogen receptor α[18,19] and progesterone receptor B.[20] Though it is thought that hypermethylation of the E-cadherin gene is associated with immunohistochemical distribution and tumor progression, tumor grade, tumor invasion, and lymph node metastasis, this has not been examined in endometrial carcinoma. In the current study, we analyzed the promoter-region CpG island methylation of the E-cadherin gene and compared it with the histopathologic features of lesions in normal endometria, endometrial hyperplasia, and endometrial carcinomas.

MATERIALS AND METHODS

Patients and Tissue Specimens

Samples of endometrial tissues were obtained from 142 women who had undergone hysterectomy or curettage at the Sapporo Medical University Hospital. Biopsy samples were obtained according to institutional guidelines (university hospital), and informed consent was obtained from patients. Normal endometrial tissue (n = 21) was taken from the unaffected endometrium of normally menstruating females with myoma or adenomyosis of the uterus. Endometrial hyperplasia tissues (n = 17) were taken from the endometrial curettage and diagnosed according to the system of the World Health Organization. As a result, three cases were found to be simple hyperplasia, nine were endometrial hyperplasia complex, and five were atypical endometrial hyperplasia. Endometrial carcinoma tissues (n = 104) were taken from modified radical hysterectomy, salpingo-oophorectomy, or selective pelvic lymphadenectomy, with para-aortic lymphadenectomy. The endometrial carcinomas were also graded according to the WHO system. This resulted in 45 cases of tumor grade G1 (well differentiated adenocarcinoma), 42 cases of G2 (moderately differentiated adenocarcinoma), 11 cases of G3 (poorly differentiated adenocarcinoma), 2 cases of serous adenocarcinoma, and 4 cases carcinosarcoma. Surgical stage was classified according to the International Federation of Gynecology and Obstetrics (FIGO) system. This result in 13 cases IA, 36 cases IB, 19 cases IC, 2 cases IIA, 3 cases IIB, 7 cases IIIA, 1 case IIIB, 19 cases IIIC, and 3 cases IVB. The samples were fixed by 10% buffered formalin for immunohistochemistry, and parts of the samples were frozen and kept at −80 °C until analysis.

Immunohistochemistry (IHC)

Tissues were fixed overnight in 10% buffered formalin, dehydrated, and embedded in paraffin. Five micrometer serial sections of each sample were used. Sections were cut, floated onto albumin coated slides, dried at 56 °C, deparaffinized in xylene, rehydrated, and washed with phosphate-buffered saline (PBS) for 15 minutes at room temperature. Specimens were treated in a microwave oven in 0.01 mol/L citrate buffer (pH 6.0) for 30 minutes at 100 °C, slowly cooled to room temperature, and then washed with PBS for 5 minutes at room temperature. After quenching endogenous peroxidase with 3% hydrogen peroxide in PBS for 10 minutes at room temperature, the sections were incubated with a blocking solution (PBS containing 5% skimmed milk) for 60 minutes at room temperature. Then the slides were incubated overnight at 4 °C with a 1:1000 dilution of anti-E-cadherin antibody (Takara, Tokyo, Japan). After several washes with PBS, they were incubated with a second antibody, a 1:200 dilution of peroxidase conjugated anti-mouse immunoglobulin (Dakopatts, Glostrup, Denmark), for two hours. The color reaction was developed by the silver intensification procedure described previously.[21] For the negative control, the same dilution of nonimmunized mouse immunogloblin was used as the first antibody.

Classification of E-cadherin Staining Pattern

All of the normal endometrium examined in the current study showed uniform, glandular staining for E-cadherin on the cell-cell borders regardless of the time in the menstrual cycle. The pattern of staining of the endometrial tumors was categorized into three types according to a previous study performed by Sakuragi et al.,[5] and staining evaluation was performed by two independent observers (T.S. and M.N.) without knowledge of clinical outcome. The positive E-cadherin expression was uniform and showed a homogeneous, fine glandular pattern (Fig. 1A) as in normal endometrium. In the heterogeneous staining pattern, only some part of the tumor showed positive staining or the cell-cell boundaries had a coarse, irregular, granular staining (Fig. 1B). In the negative staining pattern, E-cadherin expression was seen on only a few cells or it was not detected at all (Fig. 1C). The classification of the E-cadherin staining pattern was evaluated at the section close to the myometrium. Statistical analyses were performed using the Mann-Whitney test for clinocopathologic values (grade, depth of invasion,

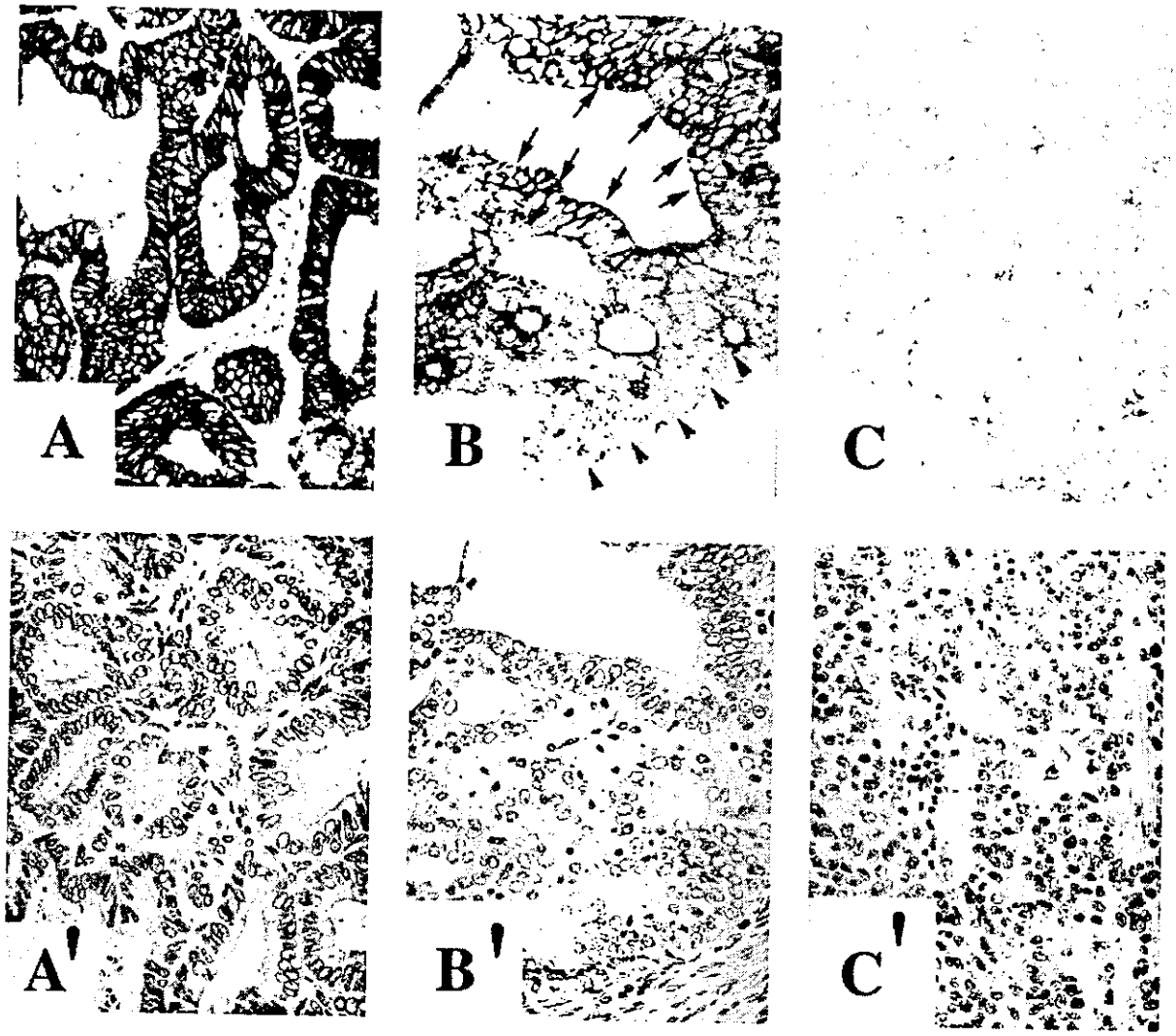

FIGURE 1. E-cadherin expression pattern in endometrial carcinoma by immunohistochemistry. A) Positive expression was characterized by uniform and homogeneous fine granular staining. B) Heterogeneous expression was defined as the pattern in which only a part of the tumor showed positive staining (arrow) and other parts negative staining (arrowhead). C) In the negative pattern, E-cadherin expression was seen in only a few cells or it was not detected at all. A', B', and C' are hematoxylin and eosin stained and serial sections of A, B, and C, respectively. Original magnification ×400.

lymph node metastasis, and surgical stage), and significance was set at $P < 0.05$.

DNA Extraction and MSP Condition

DNA was extracted from frozen samples and kept at −80 °C; 1 μg of the DNA was denatured using NaOH and treated with sodium bidulfite for 16 hours according to Herman et al.[22] Bisulfite modified DNA was amplified with specific primer pairs for the methylated DNA (E-cad-M, forward: 5'-ttaggttagagggttatcgcgt-3' and reverse: 5'-taactaaaaattcacctaccgac-3') and the unmethylated DNA (E-cad-u, forward: 5'-taattttaggttagagggttattgt-3' and reverse, 5'-cacaaccaatcaacaacaca-3'). One hundred ng of modified DNA were applied to 25 μL of polymerase chain reaction (PCR) mixture containing 2.5U AmpliTaq DNA polymerase (Takara), 1.5 mmol/L MgCl, 1 × Taq buffer, and 0.2 mmol/L four deoxynucleotide triphosphates. Thirty-eight cycles of PCR were carried out with the program of 30 seconds at 94 °C, 1 minute at 57 °C for E-cad-m or 53 °C for E-cad-u, and 1 minute at 72 °C. Parts of the PCR products were electrophoresed on 2.5% agarose gel. There were three patterns for methylation status (Fig. 2): only unmethylated, only methylated, and a mixture of methylated and unmethylated forms. Since these samples contain not only tumor cells but also surrounding connective tissue cells that originally do not express E-cadherin, we decided to group these results as unmethylated and methylated (which included both unmethylated and mixed results). Statis-

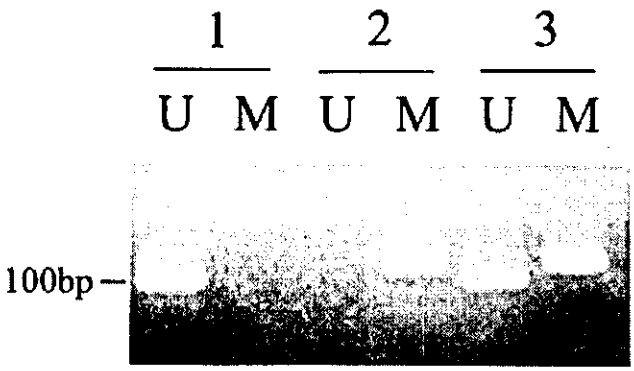

FIGURE 2. Methylation status of the promoter-region CpG island methylation of the E-cadherin gene as assayed by methylation-specific polymerase chain reaction. Bisulfite-treated DNA was amplified using two sets of primers. The M primer pair can only anneal to sequence methylated DNA before bisulfite treatment, whereas the U primer pair can only anneal to the sequence that is unmethylated. There were three patterns for methylation status: 1) only unmethylated, 2) only methylated, and 3) a mixture methylated and unmethylated forms.

tical analyses were performed using the Mann-Whitney test for clinocopathologic values (grade, depth of invasion, lymph node metastasis, and surgical stage) and significance was set at $P < 0.05$.

RESULTS

Methylation of the E-cadherin Gene and Histologic Factors in Endometrial Carcinoma

In the current study, we analyzed methylation of the E-cadherin gene and its expression by IHC using 142 endometrial tissues and their DNAs. These tissues consisted of 21 normal endometria, 17 endometrial hyperplasias, 98 endometrioid adenocarcinomas, 4 sarcomas of the endometrium, and 2 other endometrial carcinomas (Table 1). In the normal endometria, all 21 samples showed positive staining of E-cadherin by IHC. Methylation of the E-cadherin gene was not detected in any of these samples. All 17 endometrial hyperplasias, 3 simple hyperplasias, 9 adenomatous hyperplasias, and 5 atypical hyperplasias showed positive expression by IHC, but methylation of the E-cadherin gene was not detected in any of these samples.

In the 98 endometrioid adenocarcinomas, there were 45 G1 tumors, 42 G2 tumors and 11 G3 tumors. Of the 45 G1 tumor samples, 30 (66.7%) showed positive staining and 15 (33.3%) showed heterogeneous staining by IHC. Methylation of the E-cadherin gene was detected in 7 of the 45 samples (15.6%). Of the 42 G2 tumors, 8 (19.0%) had positive staining, 29 (69.0%) heterogeneous staining, and 5 (11.9%) negative staining (G1 vs. G2, $P < 0.05$). Methylation of the E-cadherin gene was found in 27 G2 tumor samples (50.0%; G1 vs. G2, $P < 0.05$). Of the 11 G3 tumors, 1 (9.1%) had positive staining, 6 (54.5%) had heterogeneous staining, and 4 (36.3%) showed negative staining (G1 vs. G3, $P < 0.05$). Methylation of the E-cadherin gene was found in nine G3 tumors (81.8%; G1 vs. G3, $P < 0.05$).

The correlations between methylation of the E-cadherin gene and the immunohistochemical findings for E-cadherin in endometrial adenocarcinomas are shown in Table 2. The ratio of samples that had methylation of the E-cadherin gene became higher with the decrease of E-cadherin expression by IHC (positive > heterogeneous > negative; positive vs. heterogeneous and heterogeneous vs. negative, $P < 0.05$). Only 2 of 39 samples (5.1%) with positive staining of E-cadherin showed methylation of the E-cadherin gene, whereas 26 of 50 samples (52.0%) with heterogeneous staining showed methylation, as did 9 of 9 (100%) with negative staining.

Surgical Stage, Hypermethylation of the E-cadherin Gene, and Immunohistologic Findings for E-cadherin in Endometrioid Adenocarcinoma

These results are shown in Table 3. The ratio of hypermethylation of the E-cadherin gene was higher in advanced stages; however, there were no significant differences among them. Immunohistologic findings for E-cadherin were similar to the results of hypermethylation, and there were no significant differences in immunohistologic findings among the surgical stages.

Depth of Myometrial Invasion and Hypermethylation of the E-cadherin Gene and Immunohistologic Findings for E-cadherin in Endometrioid Adenocarcinoma

There was also a significant correlation between the depth of myometrial invasion and hypermethylation of the E-cadherin gene (Table 4). Of the 13 samples that did not have myometrial invasion, all showed positive staining of E-cadherin by IHC and 3 of the 13 (23.1%) had methylation of the E-cadherin gene. Of the 49 samples that had invasion in less than half the myometrium, 19 (38.8%) had positive staining, whereas 28 of the 49 (57.1%) had heterogeneous staining and 2 (4.1%) were negative (negative vs. < 0.5, $P < 0.05$). Of these 49 samples, 14 (28.6%; negative vs. < 0.5, $P < 0.05$) had methylation of the E-cadherin gene. Of the 36 samples that had invasion of half or more of the myometrium, 7 (19.4%) showed positive staining, 17 (47.2%) had heterogeneous staining and 12 (33.3%) were negative (negative vs. ≥ 0.5, $P < 0.05$). Twenty of the 36 samples (55.6%; < 0.5 vs. ≥ 0.5, $P < 0.05$) had methylation of the E-cadherin gene.

Lymph Node Metastasis and Hypermethylation of the E-cadherin Gene

In the current study, the correlation between lymph node metastasis and hypermethylation of the E-cad-

TABLE 1
Pathologic Diagnosis of Endometrial Samples and Hypermethylation E-cadherin Gene and Immunohistologic Findings of E-cadherin

Pathologic diagnosis	No. of samples	Methylation	IHC of E-cadherin		
			Positive	Heterogeneous	Negative
Normal endometrium	21	0	21	0	0
Endometrial hyperplasia					
Simple	3	0	3	0	0
Adenomatous	9	0	9	0	0
Atypical	5	0	5	0	0
Endometrioid carcinoma					
G1	45	7	30	15	0
G2	42	21	8	29	5
G3	11	9	1	6	4
Serus adenocarcinoma	2	2	0	2	0
Sarcoma	4	1	0	0	4
Total	138	39	77	52	17

Methylation analysis, G1 vs. G2 and G1 vs. G3, $P < 0.05$. IHC analysis, G1 vs. G2 and G1 vs. G3, $P < 0.05$. IHC: immuno histochemistry.

TABLE 2
Immunohistochemical Finding and Methylation of E-cadherin Gene in Endometrioid Adenocarcinomas

	No. of samples	Methylation of E-cadherin gene
Positive	39	2
Heterogeneous	50	26
Negative	9	9

Positive vs. heterogeneous and heterogeneous vs. negative, $P < 0.05$

herin gene was analyzed in 98 endometrioid adenocarcinomas. As shown in Table 4, 83 of the 98 endometrioid adenocarcinomas did not have lymph node metastasis, and the other 15 cases did. Of the 83 samples that did not have lymph node metastasis, 37 (44.6%) showed positive staining of E-cadherin, 41 (49.4%) heterogeneous staining, and 5 (6.0%) negative staining, all by IHC. Twenty-eight samples (33.7%) had methylation of the E-cadherin gene. Of the 15 samples that had lymph node metastasis, 2 (13.3%) showed positive staining of E-cadherin, 9 (60.0%) heterogeneous staining, and 4 (26.7%) negative staining, all by IHC. Nine samples (60.0%) had methylation of the E-cadherin gene. No significant differences were found between samples that were positive and negative for methylation and IHC.

Additionally, we analyzed E-cadherin expression in lymph nodes that had metastasis by IHC. Of these 12 samples, one sample showed positive staining, 4 samples heterogeneous staining and 7 samples were negative. Of these seven samples that showed negative staining (Fig. 3A), three had shown heterogeneous staining in situ (Fig. 3B).

TABLE 3
Surgical Stage and Hypermethylation E-cadherin Gene and Immunohistologic Findings of E-cadherin in Endometrioid Adenocarcinoma

Surgical stage	No. of samples	Methylation	IHC of E-cadherin		
			Positive	Heterogeneous	Negative
IA	13	4	7	5	1
IB	36	8	17	18	1
IC	19	7	6	11	2
IIA	2	1	1	1	0
IIB	3	2	1	2	0
IIIA	7	4	3	3	1
IIIB	1	0	1	0	0
IIIC	15	9	2	9	4
IVB	2	2	0	1	1
Total	98	37	38	50	10

IHC: immunohistochemistry.

Hypermethylation of the E-cadherin Gene and Immunohistologic Findings for E-cadherin in Serous Adenocarcinoma and Carcinosarcoma

There were six cases without endometrioid adenocarcinoma; two were serous adenocarcinoma, and four were carcinosarcoma. The results for these samples are shown in Table 5. The two cases of serous adenocarcinoma, both of which were Stage IIIC and had the lymph node metastasis, had hypermethylation of E-cadherin, and the immunohistochemical finding was heterogeneous in both cases. In the four cases of carcinosarcoma, one case was surgical Stage IC, two Stage IIIC, and one Stage IVB. Hypermethylation was found in one sample, which was Stage IIIC, and all cases showed negative staining of E-cadherin by IHC.

TABLE 4
Relationship Between E-cadherin Methylation and IHC and Myometrial Invasion and Lymph Node Metastasis in Endometrioid Adenocarcinoma

	No. of samples	Methylation	IHC of E-cadherin		
			Positive	Heterogeneous	Negative
Myometrial invasion					
Negative	13	3	13	0	0
< 1/2	49	14	19	38	2
≥ 1/2	36	20	7	17	12
Lymph node metastasis					
Negative	83	28	37	41	5
Positive	15	9	2	9	4
Positive lymph node	12	—	1	4	7

Myometrial invasion: methylation analysis, negative vs. < half and < half vs. ≥ half, $p < 0.05$; IHC analysis, negative vs. < half and negative vs. ≥ half, $P < 0.05$.
IHC: immunohistochemistry.

DISCUSSION

Cell-cell adhesion participates in histogenesis and plays a critical role in the establishment and maintenance of cell polarity and cell society. Reduced cell-cell adhesiveness allows cancer cells to disobey the social order, resulting in destruction of the histologic structure, the morphologic hallmark of malignant tumors.[17] In cancers in vivo, particularly the diffuse type, tumor cells are dissociated throughout the entire tumor mass, lose their cell polarity, and infiltrate the stroma in a scattered manner.[17] Consistent with this concept, immunohistochemical studies have revealed that decreased E-cadherin expression is associated with tumor dedifferentiation and progression in endometrial carcinoma[5] and other tumors.[6–10] In the current study, decreased expression of E-cadherin in endometrioid adenocarcinoma was associated with tumor dedifferentiation (G1 > G2 > G3) and myometrial invasion because samples that had invasion in less than half of the myometrium showed a higher ratio of positive staining of E-cadherin than samples that had invasion of half or more of the myometrium. Though the expression of E-cadherin showed a tendency to decrease in advanced surgical stages and when there was lymph node metastasis, there were no significant differences, probably because of the small number of samples. However, of seven samples that showed negative staining in lymph nodes, three had shown heterogeneous staining in situ. The evidence suggests that E-cadherin negative cells selectively separated from the tumor and metastasized to the lymph nodes.

A recent study showed CpG methylation around the promoter region of the E-cadherin gene and induction of E-cadherin expression following treatment with the DNA methyltransferase inhibitor 5-azacytidine in human cancer cell lines lacking E-cadherin expression.[23] It was discovered that some tumor suppressor genes, including RB, VHL, p15, and p16, were inactivated as a result of

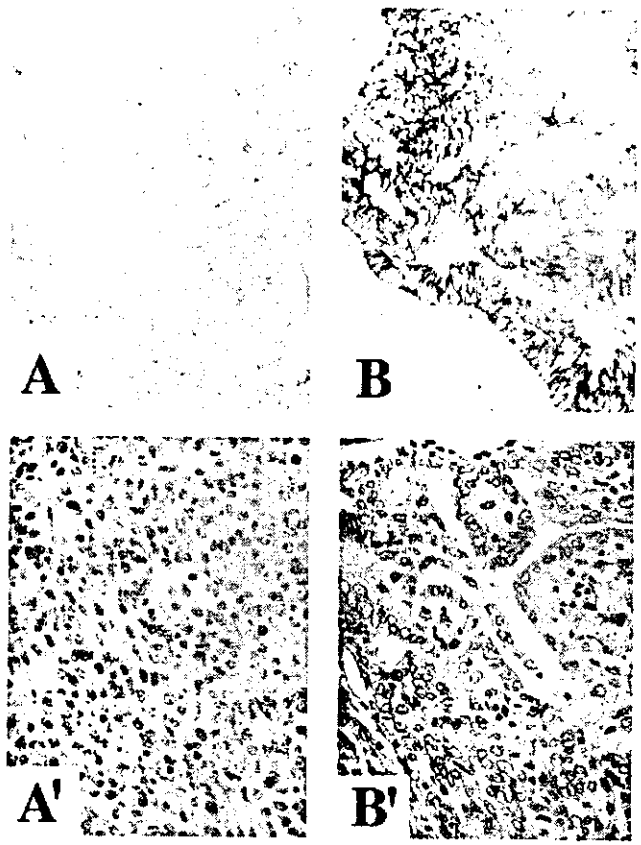

FIGURE 3. Immunohistochemical findings of E-cadherin in uterus and lymph node metastasis of endometrioid adenocarcinoma. A) E-cadherin was negative in the lymph node metastasis, B) though it showed heterogeneous localization in situ. A' and B' are hematoxylin and eosin stained and serial sections of A and B, respectively. Original magnification ×400.

TABLE 5
Clinicopathologic Finding, Hypermethylation E-cadherin Gene and Immunohistologic Findings of E-cadherin in Serus Adenocarcinomas and Carcinosarcomas

Sample	Surgical stage	Myometrial invasion	Lymph node metastasis	IHC of E-cadherin	Methylation status
Serus adenocarcinoma					
1	IIIC	>1/2	positive	heterogeneous	methylated
2	IIIC	>1/2	positive	heterogeneous	methylated
Carcinosarcoma					
1	IC	≥1/2	negative	negative	unmethylated
2	IIIC	≥1/2	positive	negative	unmethylated
3	IIIC	≥1/2	positive	negative	methylated
4	IVB	≥1/2	positive	negative	unmethylated

IHC: immunohistochemistry.

reduced expression due to CpG methylation.[24] As observed with these tumor suppressor genes, the E-cadherin invasion suppressor gene in human cancers is silenced by an epigenetic mechanism, DNA hypermethylation.[17] In the current study, hypermethylation in the promoter region of the E-cadherin gene was correlated with tumor progression, tumor dedifferentiation, and the depth of myometrial invasion. The number of samples that had methylation increased with tumor dedifferentiation, G1 (15.6%) < G2 (50.0) < G3 (81.8%). Of the samples that had invasion in less than half the myometrium, 28.1% had methylation, whereas 55.6% of samples that had invasion of half or more of the myometrium had methylation. Methylation of the E-cadherin gene correlated quite well with the immunohistochemical findings. Recently, there have been reports showing a correlation between tumor progression and methylation of the E-cadherin gene promoter in human tumors.[16,17,25–30] However, to our knowledge, this is first report that analyzed the methylation of the E-cadherin gene promoter in endometrial carcinoma.

In the current study, we analyzed six cases without endometrioid adenocarcinoma. Two cases were serous adenocarcinoma and four were carcinosarcoma. Serous adenocarcinoma[31] and carcinosarcoma[32] are known to be more aggressive, with a lower survival rate and higher rate of death from disease than the usual type of endometrial adenocarcinoma. The two cases of serous adenocarcinoma, both of which were Stage IIIC and had lymph node metastasis, had hypermethylation of E-cadherin, and immunohistochemistry was heterogeneous in both cases. The results revealed that the existence of hypermethylation and immunohistochemical findings for E-cadherin in the clinical stage, tumor invasion, and lymph node metastasis were quite similar to those of endometrioid adenocarcinomas. All four cases of carcinosarcoma, though, had negative staining of E-cadherin by IHC. Hypermethylation was found only in one sample. This may have been because the carcinosarcomas consisted mostly of sarcoma components, which originally do not express E-cadherin, and hypermethylation could not detected in them.

In the current study, methylation of the E-cadherin gene was rare in normal endometrium and endometrial hyperplasia. Endometrioid adenocarcinoma, which accounts for the majority of endometrial cancers, typifies the group of endometrial carcinomas that develop from atypical endometrial hyperplasia in the setting of excess estrogenic stimulation.[33] The evidence suggested that methylation of the E-cadherin gene might not contribute to the early events of endometrial carcinogenesis. Conversely, methylation of the E-cadherin gene was frequently found in undifferentiated tumors (81.8% of G3 tumors and 50.0% of G2 tumors). Furthermore, it was also correlated with the depth of myometrial invasion. The evidence suggested that methylation of the E-cadherin gene occurred in association with the acquisition of invasive capacity.

E-cadherin is a Ca^{2+}-dependent adhesion molecule that, in association with alpha-, beta-, and gamma-catenin, constitutes the major component of adherens junctions in vertebrates.[34] Both beta- and gamma-catenin bind directly to the cytoplasmic domain of E-cadherin, whereas alpha-catenin links the bound beta- and gamma-catenin to the microfilament network of the cytoskeleton.[35] Several reports have indicated that E-cadherin, an epithelial-specific cadherin, is a key molecule for the maintenance of epithelial integrity and of polarized states in association with alpha-, beta-, and gamma-catenin, and that the reduction of E-cadherin-mediated cell-cell adhesion favors the dispersion of cancer cells.[35] In our previous study, moderate or strong staining of beta-catenin in the nuclei was observed in 60.0% of endometrial hyperplasia samples and 30.0% of endometrial cancer samples, though the beta-catenin gene and adenomatous polyposis coli (APC) protein have neither mutation nor deletion.[36–38] This evidence implies that E-cadherin mediated cell adhesion is reduced in endometrial hyperplasias, though reduced expression of E-cadherin by hypermethylation was not found in endometrial hyperplasia.

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Combination Therapy with Granisetron, Methylprednisolone and Droperidol as an Antiemetic Prophylaxis in CDDP-Induced Delayed Emesis for Gynecologic Cancer

Satoru Sagae[a] Shin-ichi Ishioka[a] Noriyoshi Fukunaka[a]
Katsuhiko Terasawa[a] Kanji Kobayashi[a] Masaki Sugimura[a]
Yoshihiro Nishioka[a] Ryuichi Kudo[a] Masaru Minami[b]

[a]Department of Obstetrics and Gynecology, Sapporo Medical University, School of Medicine, Sapporo, and
[b]Department of Pharmacology, Faculty of Pharmaceutical Sciences, Health Science University of Hokkaido, Ishikari-Tobetsu, Japan

Key Words
Chemotherapy · Delayed emesis · Droperidol · Granisetron · Gynecologic cancer · Methylprednisolone

Abstract
Objective: To better control both acute and delayed emesis resulting from cisplatin(CDDP)-based chemotherapy for gynecological malignancies, we designed a 'cocktail therapy' (CCT) using granisetron (GRN) in combination with methylprednisolone (MPD) plus droperidol (DRP). ***Methods:*** Two crossover clinical trials were carried out to compare the efficacy and safety of (a) GRN alone (3 mg/patient) with that of GRN, MPD (250 mg/patient) and DRP (0.5 ml/patient) in 42 patients (CCT group) and (b) GRN and MPD (CMB group) with that of the CCT group in 27 patients during the first 7 days of chemotherapy, independent of the weight/body surface of the patients. One of these regimens was administered intravenously for the first 3 days of chemotherapy, in case of failure for a maximum of 5 days. ***Results:*** For acute emesis, complete protection from nausea and vomiting by the end of the 1st day was achieved in 64.3% receiving GRN and in 92.9% receiving CCT ($p < 0.01$). For delayed emesis, complete protection was best achieved in CCT on days 2–3, showing statistical significance compared to GRN treatment ($p < 0.01$). Comparing the three kinds of treatment during 7 days, the lowest protection was 38.1% in the GRN group, 51.9% in the CMB group and 72.5% in the CCT group, especially on days 2 or 3. ***Conclusions:*** The CCT combination is useful for the control of delayed and/or anticipatory emesis resulting from CDDP-based chemotherapy for women with gynecological malignancies.

Introduction

Nausea and vomiting are the most distressing adverse reactions of patients undergoing chemotherapy [1]. It is extremely important that patients with gynecologic malignancies complete their chemotherapeutic regimen. Generally, nausea and vomiting induced by anti-cancer thera-

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Satoru Sagae, MD, PhD
Department of Obstetrics and Gynecology
Sapporo Medical University, School of Medicine
South 1 West 16, Chuou-ku, Sapporo 060-0061 (Japan)
Tel. +81 11 611 2111, ext. 3373, Fax +81 11 614 0860, E-Mail sagaes@sapmed.ac.jp

py is classified into three types: acute emesis; anticipatory emesis (mental, psychological and emotional types), and delayed emesis [2]. Acute emesis occurs during the 24 h following the administration of the drug due to either direct stimulation of the chemoreceptor trigger zone or indirect stimulation of the chemoreceptor trigger zone mediated by the vagal nerve and followed by serotonin secretion at the GI tract [3]. Emotional-type emesis occurs more frequently in females. The mechanism of delayed emesis is still unclear, but it is thought to occur through the same pathways as those activated in acute and emotional emesis. Delayed emesis persists for several days, during which metabolic products of the drug and mental factors are involved [4].

The emetogenic effect of various chemotherapeutic agents differs, but particularly cisplatin (CDDP), cyclophosphamide, and doxorubicin are strongly emetic in patients with gynecologic cancer. Currently, antiemetic agents are roughly classified into four groups: benzamides, benzodiazepines, steroids, and 5-HT_3 receptor antagonists. Recently, 5-HT_3 receptor antagonists, e.g. ondansetron and granisetron (GRN), have been used in Japan. The result of a double-blind clinical study using ondansetron and GRN showed that these two drugs were effective in 40–60% of patients with acute emesis [5], and their efficacy increased to about 80% when combined with steroids [6].

The combinations of $5HT_3$ receptor antagonists with steroids have also been extensively studied and more effective than $5HT_3$ receptor antagonists alone in Japan. Various mechanisms have been proposed concerning the efficacy of a $5HT_3$ receptor antagonist combined with steroids; i.e. stimulation of the cerebral cortex, inhibition of the prostaglandin synthesis, reinforcement of the blood-brain barrier, stabilization of cell membranes, anti-inflammatory action and acceleration of the metabolism of the drug in the liver and its excretion into the urine [7, 8]. Recently, we conducted an animal study to clarify the mechanism of the effect of a $5HT_3$ receptor antagonist combined with steroids on delayed emesis [9]. In CDDP-based chemotherapy, GRN combined with steroids may be useful in suppressing delayed emesis, since mechanisms other than increased 5-HT concentration in the brain or GI tract may contribute to the occurrence of delayed emesis [9].

Typically, antiemetic efficacy for CDDP therapy will rise from the 40–50% range of complete control to 80% when dexamethasone (DEX) is added to a $5HT_3$ receptor antagonist [5–8]. However, $5HT_3$ receptor antagonists are not fully effective for delayed emesis or anticipatory emesis in gynecologic patients, even when combined with steroids. For this reason, another psychotropic agent, droperidol (DRP), is added to the combination of steroids and $5HT_3$ receptor antagonists. In the late 1980s, the combination of metoclopramide with DEX and/or DRP was shown to be clinically effective for controlling severe emesis [10]. Using $5HT_3$ receptor antagonists in a clinical setting, we have previously designed and reported a 'cocktail therapy' (called CCT) using GRN in combination with methylprednisolone (MPD) plus DRP to improve the control of both acute and delayed emesis. CCT therapy showed an excellent antiemetic effect in patients with both acute and delayed emesis [11]. In this study, we evaluated the clinical efficacy of CCT in two different crossover studies including GRN alone versus CCT therapy and GRN combined with MPD therapy (CMB) versus CCT therapy. This study demonstrates the results of the two crossover studies with GRN versus CCT and CMB versus CCT, performed at the Department of Obstetrics and Gynecology, School of Medicine, Sapporo Medical University Hospital.

Patients and Methods

Patients

From January 1996 to December 1998, 69 consecutive patients (mean age: 51.1 years, height: 154.7 cm, weight: 55.6 kg, body surface: 1.47 m^2) scheduled to receive moderately emetogenic chemotherapy were enrolled in the study. The patients had either uterine cancer or ovarian cancer. The patients received CDDP 50–70 mg/m^2 (or carboplatin >300 mg/m^2), Adriamycin 50 mg/m^2, cyclophosphamide 500 mg/m^2 as PAC regimen for endometrial cancer or ovarian cancer.

The exclusion criteria were as follows: (1) the presence of nausea and vomiting; (2) the use of antiemetic agents 1 day before the administration of chemotherapy; (3) a severe concurrent illness other than neoplasia; (4) concurrent treatment with corticosteroids or benzodiazepines; (5) a white cell count < 3,000 cells/mm^3, and (6) a platelet count of < 70,000 platelets/mm^3. Also excluded were (7) patients with a performance status > grade 3; (8) patients with marked hepatic dysfunction, and (9) patients who were unwilling or unable to comply with the treatment protocol.

Design of the Study

A comparative study with a randomized crossover design was conducted at our hospital. The study was carried out according to the provisions of the Declaration of Helsinki and was approved by the Ethics Committee of the Sapporo Medical University Hospital. Informed consent was obtained from all patients.

An overall p value of <0.05 (two-sided) was taken as indicating statistical significance.

Antiemetic Therapy

Treatment modalities were as follows: GRN alone: 3 mg GRN/patient; CMB therapy: GRN (3 mg/patient) plus MPD (250 mg/patient), and CCT therapy: GRP (3 mg/patient), MPD (250 mg/patient) and DRP (0.5 ml/patient), independent of patient weight/body surface area. One of these regimens was administered intravenously for the first 3 days of chemotherapy, in case of treatment failure for a maximum of 5 days.

On day 0, 30 min before the start of chemotherapy, patients were randomized to receive either GRN alone versus CCT therapy or CMB therapy versus CCT therapy for a randomized comparison (crossover design).

At the first treatment cycle, one group was administered CCT therapy, another GRN alone by intravenous infusion over 5 min and the other CMB therapy. All CCT-treated patients were given GRN, MPD and DRP intravenously.

In the patients treated with fractionated chemotherapeutic regimens, the above antiemetic regimen was administered daily for a maximum of 5 days (days 0–4). If nausea and vomiting occurred after the initial dose of the GRN-including regimen, subsequent doses of the GRN-including regimen were administered by slow intravenous infusion. Patients whose nausea and vomiting were not adequately inhibited by the additional doses of the GRN-including regimen were withdrawn from the study for ethical reasons and given another antiemetic chosen by their physician.

In the second treatment cycle, patients were changed to receive the alternative antiemetic treatment, one group with CCT therapy, another GRN alone and the other CMB therapy. At the end of the study, all 69 patients received CCT therapy, including 47 patients receiving GRN alone, and 27 patients CMB therapy.

Clinical Assessment

Episodes of nausea and vomiting were recorded on daily charts for the first 24 h after chemotherapy (acute effects) and for the following 6 days (delayed effects).

Nausea was recorded by patients according to their subjective symptoms. Nausea was classified by severity: 0: none, 1: mild (did not interfere with normal daily life), 2: moderate (interfered with normal daily life) and 3: severe (the patient was bedridden). The absence of nausea was defined as complete protection. The worst nausea experienced during days 2–7 was defined as the overall intensity of delayed nausea for each patient. The time to the first episode of vomiting or the beginning of any nausea was defined as the interval between the start of chemotherapy and the beginning of nausea or vomiting.

The frequency of vomiting was recorded as the number of single instances or continuous episodes of vomiting. A vomiting episode was considered to have ended when vomiting had ceased for at least 1 min. Complete protection was defined as the absence of vomiting episodes, while major protection was defined as one or two episodes. Failure of treatment was defined as three or more episodes. Complete or major protection was considered to indicate successful treatment.

Adverse events other than episodes of vomiting or nausea were recorded on the daily charts by patients in response to questioning. Any adverse event, as well as the presence of nausea or vomiting, from day 8 to the start of the subsequent cycle of chemotherapy was also recorded.

A patient who had three or more vomiting episodes or severe nausea (or both) could receive oral tablets of metoclopramide (5 mg) or another 5-HT_3 receptor antagonist, such as ondansetron.

Statistical Analysis

Analyses of nausea and vomiting were performed separately for day 1 (acute effects) and for days 2–7 (delayed effects).

Statistical analysis was carried out to present the data as a crossover trial. Where appropriate, statistical analysis of the parallel trial data was used to confirm the crossover results. In the crossover analysis, a two by two χ^2 test was used to test significant differences among the groups during the 7-day period. Fisher's exact test was used to evaluate the balance of prognostic factors and to compare each adverse event among the three groups.

Results

Patient Characteristics

The characteristics of the 69 patients in this comparative study are shown as patients with GRN alone, CMB therapy, and CCT therapy in table 1. The patients in each group were well matched for age, height, weight, body surface, performance status, primary disease and metastatic disease. Any brain tumor or GI obstruction was not detected in our patients during these studies.

Acute Emesis

Table 2 shows the data on acute nausea, vomiting, or both, with GRN alone and CCT therapy or CMB therapy and CCT therapy. The CCT therapy was significantly more effective than GRN, whereas CCT and CMB therapies had similar effects (column 2, table 2, the second trial).

Complete protection from nausea at the end of the 1st day was achieved in 30 of the 42 patients receiving GRN alone (71.4%); in 40 of the 42 receiving CCT therapy (95.2%) in the first trial, and in 20 of the 27 patients receiving the CMB therapy (74.1%); and in 25 of the 27 receiving the CCT therapy (92.6%) in the second trial. Response to treatment was significantly better in the 42 patients receiving the CCT therapy, especially after 12–24 h ($p < 0.01$). For the first 6 h, there is no significant difference among the three antiemetic therapies. Twelve patients had nausea in the group treated with GRN alone, but only 2 patients had nausea in the CCT group in the first trial. In the second trial, 7 patients had nausea in the CMB group, but only 2 in the CCT group.

Complete protection from vomiting was also achieved in a larger number of patients receiving CCT therapy (41 of 42, as compared with 31 of 42 in the GRN alone group; $p < 0.05$) at 12–18 h during the 1st day of the first trial, but a similar tendency was not significant in the second trial between the CMB and CCT therapies.

Table 1. Characteristics of 69 patients receiving antiemetic treatment with GRN alone, CMB therapy, and CCT therapy in different randomized comparative studies

		GRN alone/CCT therapy (n = 42)			CMB therapy/CCT therapy (n = 27)			Total patients (n = 69)	
Age, years		52.2 ± 12.0			50 ± 12.1			51.1 ± 12.0	NS
Height, cm		154.7 ± 5.6			154.8 ± 6.0			154.7 ± 5.7	NS
Weight, kg		57.2 ± 10.7			53 ± 6.5			55.6 ± 9.5	NS
Body surface, m^2		1.47 ± 0.1			1.48 ± 0.1			1.47 ± 0.1	NS
Performance status	0	34			25			59	
	1	6			2			8	
	2	2			0			2	
Primary site	Ovary	23			15			38	
	Uterus	19			12			31	
Clinical stage	I	17	11[a]	6[b]	11	5[a]	6[b]	28	
	II	7	2[a]	5[b]	5	3[a]	2[b]	12	
	III	12	6[a]	6[b]	10	7[a]	3[b]	22	
	IV	6	4[a]	2[b]	1	0[a]	1[b]	7	
Metastatic disease	Present	18			12			30	
	Absent	24			15			39	

[a] Ovary.
[b] Uterus.
NS = Not significant.

Table 2. Percentages of patients with complete protection from acute nausea, vomiting, or both

Complete protection	GRN alone (n = 42)		CCT therapy (n = 42)		p value χ^2 test	CMB therapy (n = 27)		CCT therapy (n = 27)		p value χ^2 test
	n	%	n	%		n	%	n	%	
Nausea										
<6 h	40	95.2	42	100	NS	26	96.3	27	100	not done
6–12 h	38	90.5	41	97.6	NS	27	100	26	96.3	NS
12–18 h	32	76.2	40	95.2	0.004	20	74.1	23	85.2	NS
18–24 h	30	71.4	40	95.2	0.003	20	74.1	25	92.6	NS
Vomiting										
<6 h	41	97.6	42	100	NS	27	100	27	100	not done
6–12 h	37	88.1	41	97.6	NS	27	100	26	96.3	NS
12–18 h	31	73.8	41	97.6	0.015	21	77.8	24	88.9	NS
18–24 h	32	76.2	39	92.9	NS	20	74.1	23	85.2	NS
Nausea and vomiting										
<6 h	40	95.2	42	100	NS	26	96.3	27	100	NS
6–12 h	34	81	41	97.6	0.013	27	100	26	96.3	NS
12–18 h	28	66.7	40	95.2	<0.001	19	70.4	23	85.2	NS
18–24 h	27	64.3	39	92.9	0.001	19	70.4	23	85.2	NS

NS = Not significant.

Table 3. Percentages of patients with complete protection from vomiting, nausea, or both on days 2 through 7

Complete protection	GRN alone (n = 42)		CCT therapy (n = 42)		p value χ^2 test	CMB therapy (n = 27)		CCT therapy (n = 27)		p value χ^2 test
	n	%	n	%		n	%	n	%	
Nausea										
Day 2	19	45.2	36	85.7	<0.001	16	59.3	23	85.2	0.033
Day 3	21	50	35	83.3	0.001	17	63	24	88.9	0.025
Day 4	31	73.8	39	92.9	0.019	17	63	27	100	<0.001
Day 5	36	85.7	41	97.6	0.048	18	66.7	27	100	0.001
Day 6	38	90.5	40	95.2	NS	22	81.5	26	96.3	NS
Day 7	40	95.2	41	97.6	NS	24	88.9	27	100	NS
Vomiting										
Day 2	23	54.8	31	73.8	NS	17	63	21	77.8	NS
Day 3	27	64.3	33	78.6	NS	19	70.4	19	70.4	NS
Day 4	33	78.6	35	83.3	NS	22	81.5	21	77.8	NS
Day 5	40	95.2	35	83.3	NS	23	85.2	25	92.6	NS
Day 6	40	95.2	39	92.9	NS	24	88.9	26	96.3	NS
Day 7	40	95.2	40	95.2	NS	25	92.6	26	96.3	NS
Nausea and vomiting										
Day 2	16	38.1	30	71.4.7	0.002	15	55.6	20	74.1	NS
Day 3	17	40.5	31	73.8	0.002	14	51.9	19	70.4	NS
Day 4	27	64.3	33	78.6	NS	16	59.3	21	77.8	NS
Day 5	36	85.7	35	83.3	NS	18	66.7	25	92.6	0.018
Day 6	37	88.1	38	90.5	NS	22	81.5	25	92.6	NS
Day 7	39	92.9	39	92.9	NS	24	88.9	26	96.3	NSl

NS = Not significant.

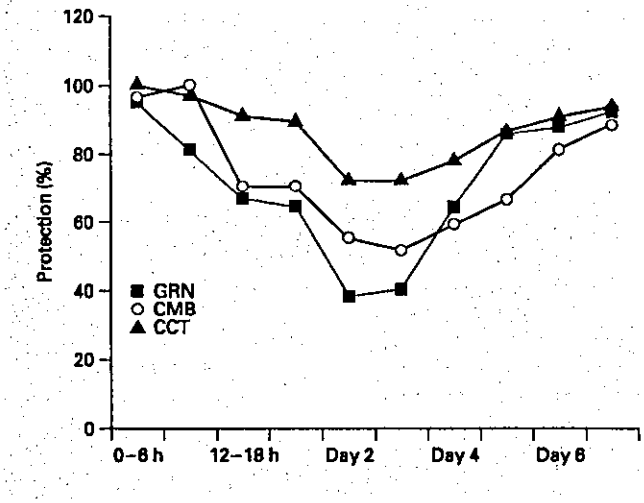

Fig. 1. Complete protection from both nausea and vomiting for 7 days with respect to treatment.

Complete protection from both nausea and vomiting was also achieved in a significantly larger number of patients treated with the CCT therapy (39 of 42, as compared with 27 of 42 in the GRN group; $p < 0.01$). The treatment was successful in 42 patients in the CCT group, especially at 12–24 h ($p < 0.01$). In the second trial, we did not observe a significant difference in the complete protection from both nausea and vomiting between the CMB and CCT groups.

Delayed Emesis

We also evaluated delayed nausea, vomiting, or both occurring during days 2–7 in all 69 patients (table 3). The number of patients with complete protection from nausea on days 2–5 was significantly higher in the CCT group compared to the group receiving GRN alone or CMB. On days 6 and 7, the efficacy of the two antiemetic treatments did not differ significantly. Complete protection from vomiting was not significantly different between the therapies of the two trials, but both nausea and vomiting were completely prevented with CCT on treatment days 2 and 3 in the first trial ($p < 0.01$).