the other groups. The incidence of postoperative interstitial complications with adjuvant RT was reported as 35.5% by Els *et al.*[24] and 48% by Bye *et al.*[25] Thus, radical hysterectomy followed by adjuvant RT results in a higher rate of bowel complications than CT.

Because of the non-randomized design of the study, we cannot draw any conclusion regarding the respective effects of the two adjuvant treatment modalities on overall survival. However, we can conclude that the effect of CT on disease-free survival was no worse than that of RT for patients without multiple LNM, and was associated with fewer bowel complications. Therefore, we believe that it is worth considering a prospective randomized trial of CT versus RT as an optional adjuvant therapy to patients with intermediate risk factors for recurrence.

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Midkine and its clinical significance in endometrial carcinoma

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(Received November 17, 2007/Revised January 24, 2008; January 30, 2008/Accepted January 31, 2008/Online publication April 14, 2008)

Midkine (MK) is a secreted heparin-binding growth factor. Several types of human cancer have increased MK expression with elevated serum levels. The purpose of this study was to determine whether MK was expressed in endometrial carcinoma and to evaluate the clinicopathological significance of serum MK in patients with endometrial carcinoma. Immunohistochemical expression of MK was evaluated in 85 endometrial carcinoma samples and 33 controls. MK expression was significantly higher in the carcinomas than in normal endometrium (*P* < 0.001). Interestingly, MK expression was highest at the margins of invasion and low in the superficial areas of the tumor samples. Using ELISA, we compared serum MK concentration in 120 endometrial carcinoma patients with the concentration in 46 patients with benign gynecologic tumors. Serum MK value in patients with cancer was significantly higher than that in the patients with benign diseases (*P* = 0.01). Patients with positive lymph node metastasis or recurrence, or cancer death, had a higher serum MK level (*P* = 0.008, *P* = 0.009, respectively). In conclusion, MK immunoreactivity in endometrial carcinoma is significantly higher than in normal endometrium. Additionally, preoperative serum MK levels are significantly correlated with prognosis and the presence of lymph node metastasis. Thus, MK may be a useful serum biomarker for identifying high risk patients of endometrial carcinoma. (*Cancer Sci* 2008; 99: 1125–1130)

Endometrial carcinoma is one of the most common female pelvic malignancies worldwide, and its incidence has recently increased in Japan.[1,2] As approximately 80% of endometrial carcinomas are diagnosed at an early stage when surgery is curative, they carry a better prognosis than other cancers. However, advanced or recurrent cases tend to respond poorly to conventional treatments such as radiation, chemotherapy, or hormonal therapy, and as a result carry a poor prognosis. Identification of additional prognostic markers could help detect patients at a high risk of relapse or death from the disease.

Clinical, biological, and epidemiological findings all suggest that prolonged or unopposed estrogenic stimulation increases the risk of type I endometrial carcinoma. The initiation and progression of type I endometrial carcinoma, however, are poorly understood at a molecular level. We previously studied the gene expression profile of endometrioid adenocarcinoma, and identified 24 genes that had at least a 1.5-fold increased expression in both well (grade 1) and poorly (grade 3) differentiated endometrioid adenocarcinoma compared to normal endometrium (unpublished data). MK was identified as one of the up-regulated genes. Though MK expression has been reported in many human cancers, it has not been studied in endometrial carcinoma. Therefore, we focused our subsequent experiments on the actions of MK.

MK is a secreted, heparin-binding growth factor. It is a 13-kDa protein rich in basic amino acids and cysteine.[3,4] MK is highly expressed in the mid-gestational period during embryogenesis, and is involved in tooth, lung, kidney, and bone development. In the adult, MK has a very restricted pattern of expression. The highest transcript levels are in the intestine with low levels in the cerebellum, thyroid, kidney, bladder, lung alveoli, colon, stomach, and spleen.[5] The pathophysiological effects of MK include the oncogenic transformation of fibroblasts, antiapoptotic activity, and angiogenic activity.[6–8] MK mRNA levels and protein expression are frequently elevated in various human carcinomas of the breast, lung, esophagus, colon, ovary, urinary bladder, and prostate; and glioblastomas, neuroblastomas, and Wilms' tumor.[9–18] Furthermore, MK concentrations in serum are also elevated in various carcinomas.[19–22] To our knowledge, however, no study has focused on the clinicopathological significance of MK expression in human endometrial carcinoma. The purpose of this study was to determine whether MK was expressed in endometrial carcinoma, and whether differences existed between the expression level in cancer and levels in benign gynecologic conditions. We also explored whether correlations existed between MK expression and clinicopathological features.

Materials and Methods

Tissue and serum samples. Eighty-five endometrioid endometrial carcinomas (37 well differentiated, 25 moderately differentiated, 23 poorly differentiated; 55 stage I, 16 stage II, 11 stage III, three stage IV) were retrieved from the surgical pathology files of Tohoku University Hospital, Sendai, Japan for immunohistochemical analysis. The controls were selected from patients who underwent hysterectomy for benign gynecologic diseases without any personal cancer history from April 1996 to March 2004. The median follow-up time for patients whose samples were examined immunohistochemically was 60 months (range, 2–148 months). The disease-free and overall survival times of the patients were calculated from the time of initial surgery to recurrence or death, or the date of last contact. The survival times of patients still alive or lost to follow-up were censored in December 2004. Serum samples were obtained from 120 patients with endometrial carcinoma (66 well differentiated, 16 moderately differentiated, 12 poorly differentiated, 26 other histological type; 80 stage I, 11 stage II, 17 stage III, 12 stage IV) and from 45 patients with non-malignant gynecologic diseases at Tohoku University Hospital from April 2002 to January 2007. None of the patients examined had received radiation, hormonal therapy, or chemotherapy prior to surgery. The median follow-up time for the patients whose serum was tested for MK was 91 months (range, 1–166 months). The

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doi: 10.1111/j.1349-7006.2008.00796.x
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survival times of patients still alive or lost to follow-up were censored in August 2007. The protocol for this study was approved by the Ethics Committee at Tohoku University School of Medicine.

Total RNA extraction from endometrial tissues and cDNA synthesis. All tumor and normal specimens were frozen in liquid nitrogen and stored at –80°C. Total RNA was extracted from normal endometrium and carcinoma tissues, using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A reverse transcription kit, SuperScript III RT (Invitrogen, Carlsbad, CA, USA), was used for the synthesis of cDNA.

Real-time reverse transcription–polymerase chain reaction (RT-PCR). Real-time PCR was carried out using the LightCycler System (Roche Diagnostics, Mannheim, Germany). cDNAs of known concentrations for target genes and the housekeeping gene, ribosomal protein L13a (RPL13A) were used to generate standard curves for determining the quantity of target cDNA transcripts. The mRNA level in each case was represented as a ratio with RPL13A.[23] The PCR thermal profile for MK was: initial denaturation at 95°C for 10 min followed by 32 amplification cycles of denaturation at 95°C for 10 s, annealing at 68°C for 10 s, and elongation at 72°C for 12 s; and for RPL13A, initial denaturation at 95°C for 10 min followed by 30 amplification cycles of denaturation at 95°C for 12 s, annealing at 68°C for 10 s, and elongation at 72°C for 12 s.

The primer sequences used in our study were: 5′-CCA AGA CCA AAG CAA AGG-3 and 5′-GGC AGG GCA TGA TTG ATT-3′ for MK; 5′-CCT GGA GGA GAA GAG GAA AGA GA-3′ and 5′-TTG AGG ACC TCT GTG TAT TTG TCA A-3′ for RPL13A.

Immunohistochemistry. After deparaffinization and rehydration in graded alcohol, antigen retrieval for MK immunostaining was done by heating the sections in a 600-W microwave for 20 min in 10 mM trisodium citrate buffer, pH 7.0. The sections were then blocked with normal goat serum for 30 min at room temperature, followed by incubation with chicken antihuman MK antibody (given by K.K.) overnight at 4°C. The dilution of the primary antibody used in this study was 1/250. The slides were incubated in 99.7% methanol containing 0.3% hydrogen peroxide at room temperature for 30 min to inhibit endogenous peroxidase. They were then incubated with biotin-conjugated rabbit antichicken IgG (ICN Pharmaceuticals, Aurora, OH, USA) at room temperature for 30 min, followed by incubation with peroxidase-conjugated streptavidin for 30 min at room temperature, using a Histofine Kit (Nichirei, Tokyo, Japan). The antigen-antibody complex was visualized with a 3, 3′-diaminobenzidine solution (1 mmol/L 3, 3′-diaminobenzidine, 50 mmol/L Tris-HCl [pH 7.6], 0.006% H2O2) and counterstained with hematoxylin. Serous adenocarcinoma of the ovary was employed as a positive control for MK immunostaining.[13] The primary antibody was replaced with phosphate-buffered saline (PBS) as a negative control. Samples were considered negative if none of the cells stained for MK. Very weak positive was defined as less than 5% staining, weak positive as 5–25% staining, moderate positive as 25–50% staining, and strong positive as more than 50% staining. Slides were then numerically scored based on immunoreactivity. A score of 0 was negative, 1 very weak, 2 weak, 3 moderate, and 4 strong positive.

ELISA for human MK. An ELISA for human MK was performed as described previously.[22] Briefly, human MK was produced using Pichia pastoris GS115 by transfection with a human MK expression vector, which was constructed into pHIL-D4 (Invitrogen). This yeast-produced human MK was used to immunize rabbits and chickens to raise antibodies. The rabbit antihuman MK antibody (50 mL of 5.5 mg/mL in 50 mM Tris HCl [pH 8.2], 0.15 M NaCl, 0.1% NaN_3) was coated onto the wells of microtiter plates (Polysorpplates; Nunc, Rochester, NY, USA) for 20 h at room temperature. After washing with 0.05% Tween 20 in PBS, the wells were blocked with 300 mL of 0.1% casein, 0.01% Microcide I (aMReSCO) in PBS for 20 h at 37°C. Plasma samples (10 mL each) were mixed with 100 mL of 50 mM Tris HCl (pH 8.4), 0.5 M KCl, 0.1% casein, 0.5% bovine serum albumin, 0.01% Microcide I, and 0.1 mg/mL peroxidase-labeled chicken antihuman MK antibody. Aliquots of 50 mL of this mixture were added to wells prepared as described above, and subjected to chromogenic detection at OD450 using tetramethylbenzidine as the substrate. This ELISA system shows linearity from 0 to 4 ng/mL of MK, and there is no crossreaction with Pleiotrophin.[22]

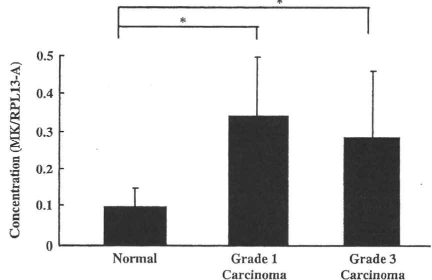

Fig. 1. Midkine (MK) mRNA expression levels in normal endometrial tissues and endometrial carcinoma tissues measured by reverse transcription–polymerase chain reaction (RT-PCR). MK mRNA expression levels in carcinoma tissues were significantly higher than in normal endometrial tissues ($P < 0.001$, Mann–Whitney test).

Statistical analysis. mRNA levels and serum concentrations of MK were compared using the Mann–Whitney test. Immunoreactivities for MK were compared using a Student's t-test. P-values less than 0.05 were considered significant.

Results

MK was expressed at higher levels in endometrioid adenocarcinoma tissues than in normal endometrium samples. To validate the microarray-based MK expression difference, we performed real-time RT-PCR using cDNA from 10 normal endometrium specimens and 20 carcinoma specimens; 10 were grade 1 and 10 were grade 3. The quantitative mRNA expression levels of MK were significantly higher in the endometrioid adenocarcinomas than in normal endometrium samples. However, there was no difference in the expression level between grade 1 and grade 3 (Fig. 1).

We then confirmed the high expression of MK in carcinoma tissues not only at the mRNA level but also at the protein level by immunohistochemical staining. The intensity of MK immunostaining in tissues is summarized in Table 1. As shown in Figure 2, MK protein was predominantly expressed in the epithelial cytoplasm with little nuclear expression. Positive staining for MK was scarcely detected in the stroma. In both normal proliferative and secretory phase endometrium samples, MK expression in the basal layer was significantly stronger than in the functional layer or endometrial stroma ($P < 0.001$, t-test) (Table 1 and Fig. 2c–f). No significant difference in protein expression was detected between the endometrial stroma and the functional layer in either the proliferative or the secretory phase. MK immunoreactivity at the basal layer tended to be stronger in

doi: 10.1111/j.1349-7006.2008.00796.x
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Table 1. Midkine protein expression in normal and endometrial cancer tissues by immunohistochemistry (mean ± SD of immunostaining score)

Normal	n	Endometrial stroma	Functionalis	Basalis	
Total	33	0.41 ± 0.56	0.62 ± 0.89	*1.72 ± 1.17*	*P** < 0.001*
Proliferative	21	0.35 ± 0.61	0.62 ± 0.86	1.35 ± 1.18	
Secretory	12	0.50 ± 0.52	0.67 ± 0.98	2.17 ± 0.94	
Carcinoma	n	Endometrial stroma	Superficial area	Invasive area	
Total	85	0.38 ± 0.56	0.81 ± 0.78	*2.66 ± 0.79*	
G1	37	0.41 ± 0.55	1.00 ± 0.77	2.69 ± 0.82	
G2	25	0.32 ± 0.56	0.60 ± 0.71	2.56 ± 0.77	
G3	23	0.39 ± 0.58	0.75 ± 0.79	2.74 ± 0.81	

**P*-value, *t*-test.

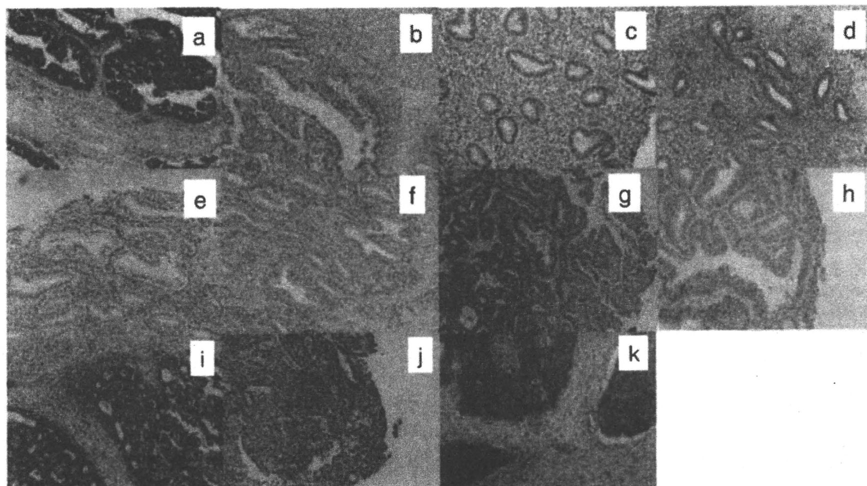

Fig. 2. Representative panels of immunohistochemical staining with anti-midkine (MK) protein antibody. (a) Positive control, (b) negative control, (c) proliferative phase (functionalis), (d) proliferative phase (basalis), (e) secretory phase (functionalis), (f) secretory phase (basalis), (g) transitional area of endometrial carcinoma grade 1, (h) superficial area of endometrial carcinoma grade 1, (i) invasive area of endometrial carcinoma grade 1, (j) superficial area of endometrial carcinoma grade3, (k) invasive area of endometrial carcinoma grade 3.

the secretory phase than in the proliferative phase ($P = 0.09$, *t*-test). Interestingly, MK expression was strongest at the margins of invasion and low in the superficial layers of the tumor samples (Fig. 2g–j). MK expression was significantly higher in the carcinomas than in the basal area of the normal endometrium ($P < 0.001$, *t*-test) (Table 1). No statistical correlation was detected between grade 1 and grade 3 endometrioid adenocarcinoma. MK immunoreactivity was not associated with any clinicopathological features including histological grade, depth of myometrial invasion, the presence of lymph node metastasis, or prognosis.

Serum MK protein concentration was higher in patients with endometrial carcinoma than in patients with benign gynecologic diseases. We measured serum MK protein concentrations with ELISA. Serum MK values for the patients with endometrial carcinoma was significantly higher than those for patients with benign gynecologic diseases ($P = 0.01$, Mann–Whitney test). The data suggest that MK protein is not only expressed in cancer tissues but also secreted into the sera at higher levels in endometrial carcinoma patients. To test whether the serum MK level could be used to discriminate endometrial carcinoma from benign disease, we set various cut-off values and classified the cases based on their MK values. Serum MK level had a high false negative ratio, thereby limiting its use in clinical applications.

A higher serum MK protein concentration was correlated with the presence of lymph node metastases and prognosis of endometrial carcinomas. We calculated the mean serum MK concentrations of cancer patients categorized by clinicopathological features. Results of the associations between clinicopathological parameters and serum MK levels are summarized in Table 2. Serum MK concentration was not associated with age, histological grade, or lymphovascular invasion. Although serum MK had a tendency to be lower in stage I–II or no myometrial

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Table 2. Serum midkine (MK) levels and clinicopathological factors in endometrial carcinomas

Clinicopathological factors		N (%)	MK concentrations (Mean ± SD)	P*-values
Age	50 =	25 (21)	104 ± 253	0.111
	50 <	95 (79)	81 ± 113	
Histological grade	Grade1	66 (55)	82 ± 169	0.455
	Grade2	16 (13)	64 ± 97	
	Grade3	12 (10)	144 ± 112	
	Others	26 (22)	76 ± 143	
Stage	I–II	91 (76)	71 ± 157	0.054
	III–IV	29 (24)	133 ± 159	
Myometrial invasion	None	19 (16)	46 ± 76	0.074
	< 1/2	58 (48)	79 ± 178	
	= 1/2	40 (33)	100 ± 130	
	Unknown	5 (4)	183 ± 201	
Lymphovascular invasion	Negative	83 (69)	75 ± 153	0.720
	Positive	35 (29)	90 ± 139	
	Unknown	2 (2)	400 ± 33	
Lymph node metastasis	Negative	103 (86)	73 ± 142	0.008
	Positive	5 (4)	253 ± 246	
	Unknown	12 (10)	131 ± 161	
Prognosis	Non-recurrence	102 (85)	71 ± 142	0.009
	Recurrence or death	18 (15)	172 ± 184	

*P-value, Mann–Whitney test.

invasion, the difference was not statistically significant ($P = 0.054$, $P = 0.072$). Interestingly, the patient group with positive lymph node metastasis had a higher level of serum MK ($P = 0.008$, Mann–Whitney test). Patients with recurrence or cancer related death had significantly higher serum levels of MK protein than those without recurrence ($P = 0.009$).

Discussion

This is the first report showing that mRNA levels and protein expression of MK in endometrial carcinoma are significantly higher than in normal endometrium. Additionally, serum MK levels in endometrial carcinoma patients were significantly elevated relative to levels in patients with benign gynecologic diseases. Although MK is overexpressed in various human malignant tumors, its effects on tumor growth and progression are not fully understood. Growth of mouse colorectal carcinoma cells is inhibited by antisense midkine oligo DNA.[24] Transfection of the breast carcinoma line MCF-7 with MK accelerates tumor growth and increases tumor vascularity after cell implantation in nude mice.[25] MK also rescues Wilms' tumor cells from cisplatin-induced apoptosis.[26] These effects are likely mediated by signaling via phosphatidylinositol-3-kinase and mitogen-activated kinase.[27] Taken together these biological data support the hypothesis that MK plays an important role in oncogenesis and tumor progression.

Despite the increased MK immunoreactivity in endometrial carcinomas, there was no relationship between immunoreactivity and clinicopathological features. This was surprising since high MK immunoreactivity significantly correlates with worse clinical outcome of neuroblastomas,[17] urinary bladder cancer,[14] gastrointestinal stromal tumor,[28] oral squamous cell carcinomas,[29] and pancreatic cancer.[30] Interestingly, in esophageal carcinoma, MK is more intensely expressed in well-differentiated tumors than in poorly differentiated tumors.[11] A noteworthy immunohistochemical finding in this study was that the intensity of MK protein expression was not the same across different areas within a single tissue sample. MK expression in normal endometrium was higher in the basalis than in the functionalis. It was highly expressed at the margin of invasion but not in the superficial areas of the cancer specimens. To confirm that these findings were not due to the unequal localization of antibody, endometrial biopsy samples from cancer patients were also immunostained. These superficial specimens all demonstrated weak expression (data not shown). The MK immunohistochemical findings in normal endometrium were inconsistent with the previously reported pathophysiological effects of MK. MK is involved in angiogenesis and antiapoptosis. Microvessel density in normal endometrium, however, is not significantly different between the functionalis and basalis,[31] and apoptotic cells are equally distributed on each layer.[32] Donoghue *et al.* reported that lymphatic vessel density (LVD) is higher in the basalis than in the functionalis across the menstrual cycle.[31] In this study, the distribution of lymphatic vessels is consistent with the diversity of MK immunoreactivity across the menstrual cycle. Rogers *et al.* suggested that unknown lymphangiogenic growth factors may be involved in normal endometrium, since no difference is observed in immunostaining intensity for the vascular endothelial growth factor (VEGF)-C or VEGF-D between the functionalis and basalis.[33] We speculate that MK would be a candidate molecule for lymphangiogenesis in normal endometrium. In endometrial adenocarcinoma, the peritumoral LVD is higher compared with the LVD within the tumor and in normal endometrium, which also correspond to MK immunoreactivity. These observations suggest a role for MK in lymphangiogenesis in endometrial adenocarcinoma.

Since MK is a secretory protein, it could potentially be used to screen for and monitor the progression of endometrial carcinoma in a manner similar to cancer antigen (CA)–125 for ovarian cancer. An elevated serum MK level is detected in more than 80% of human adult carcinomas, and its level decreases when the tumor is resected.[19] A high serum MK level is associated with higher stage and disease progression in gastric cancer,[21] with tumor size in esophageal cancer,[20] and with progression in neuroblastoma.[22] As shown in Figure 3, serum MK was significantly elevated in patients with endometrial carcinoma compared with patients with non-malignant gynecologic diseases ($P = 0.014$). Regarding the relationship between serum MK concentration and clinicopathological features in patients with endometrial carcinoma, statistical differences were seen in both lymph node metastasis and prognosis. Our observations are consistent with another recent study in esophageal carcinoma.[34] In

doi: 10.1111/j.1349-7006.2008.00796.x
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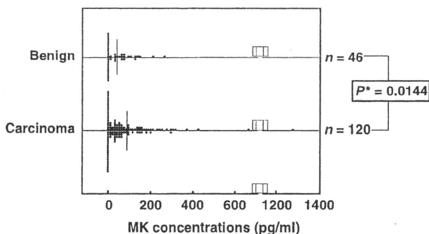

Fig. 3. Serum midkine (MK) protein concentrations from patients with benign gynecologic tumors or endometrial carcinoma. MK protein levels were measured by ELISA. The serum concentration for the carcinoma patients was significantly higher than that for the benign patients ($P = 0.014$, Mann–Whitney test).

esophageal squamous cell carcinoma, serum MK is a good marker of lymph node metastasis that correlates with serum levels of VEGF-C. Lymph node metastasis is a critical prognostic factor in endometrial carcinoma, and myometrial invasion and histological grade are correlated strongly with lymph node metastasis.[35,36] Thus, preoperative serum MK levels might prove to be useful for selecting high risk patients or predicting prognosis.

In conclusion, MK immunoreactivity in endometrial carcinoma is significantly higher than in normal endometrium. Additionally, preoperative serum MK levels are significantly correlated with prognosis and the presence of lymph node metastasis. Further, larger, prospective studies with longer follow-up periods are needed to fully understand the role of MK in endometrial carcinoma carcinogenesis.

Acknowledgments

We are grateful to Dr Jun-ichi Akahira and Dr Takashi Suzuki for helpful suggestions. This study was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas, a Grant-in-Aid for Scientific Research (B) and (C), a Grant-in-Aid for Young Scientists (B), and a Grant-in-Aid for Exploratory Research, from the Ministry of Education, Science, Sports, Culture, and Technology of Japan; a Grant-in-Aid from the Ministry of Health, Labor, and Welfare of Japan; the 21st Century COE Program Special Research Grant (Tohoku University) from the Ministry of Education, Science, Sports, Culture, and Technology of Japan; a Grant-in-aid from the Kurokawa Cancer Research Foundation; and the Uehara Memorial Foundation.

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doi: 10.1111/j.1349-7006.2008.00796.x
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doi:10.1111/j.1447-0756.2008.00818.x *J. Obstet. Gynaecol. Res. Vol. 34, No. 4: 567–573, August 2008*

Expression of melatonin receptor (MT1) and interaction between melatonin and estrogen in endometrial cancer cell line

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Abstract

Aim: To determine the receptor subtypes of melatonin in estrogen receptor-positive endometrial cancer cell line, Ishikawa, and the influence of melatonin on chemosensitivity.

Methods: To confirm the subtype of melatonin on Ishikawa cells, cells were treated with melatonin alone and with antagonists against melatonin receptor luzindole and 4-phenyl-2-propionamidotetralin (4-P-PDOT). Expression of MT1/MT2 mRNA was analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR). Immunocytochemistry of MT1/MT2 was also performed. The effect of melatonin against expression of MT1, MT2, and ERα-receptors mRNA was compared with RT-PCR. To determine whether melatonin enhances the effect of anticancer agents, chemosensitivity test was performed with or without melatonin.

Results: Our study revealed that Ishikawa cells express MT1 by both RT-PCR and immunocytochemistry. In contrast, expression of MT2 mRNA was not found. Furthermore, ERα mRNA expression was attenuated at melatonin level of 1×10^{-9} M. Chemosensitivity test revealed that melatonin enhanced anti-tumor effects of paclitaxel among anticancer drugs tested.

Conclusion: Based on the above results, MT1 receptor, but not MT2, is expressed in Ishikawa cells. It was also revealed that the cytostatic effect of melatonin is partly an action mediated by MT1 receptor, and attenuation of ERα expression was predicted as the mechanism of action. Clinical application of melatonin to biochemotherapy might be also expected.

Key words: endometrial cancer, estrogen, melatonin, MT1.

Introduction

Melatonin is a hormone synthesized from tryptophan in the pineal gland and has a variety of actions, such as circadian rhythm regulation, seasonal reproduction, immune mechanism integration and inhibition of tumorigenesis.[1] While the anticancer action of melatonin has been reported in a variety of solid cancers, there have been numerous reports on breast cancer, particularly on signaling mechanisms in the anticancer action of melatonin[2] and interactions between melatonin and estrogen in estrogen receptor-positive breast cancer.[3,4] Melatonin has been thought to control various second messengers in signaling mechanisms through receptors; however, although there are reports on the involvement of intracellular Ca^{2+} in mechanisms not mediated by a receptor, there is no well-established theory.[5]

Endometrial cancer, which has recently been on the increase, is classified into type 1, which occurs in younger people aged 40 years or younger; and type 2, which occurs in the elderly. Type 1 is induced by a

Received: August 31 2007.
Accepted: November 13 2007.
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persistent estrogen dominant endocrine environment due to ovulation disorder caused by hyperprolactinemia and obesity, etc.[6] Hormone therapy has been performed infrequently and its efficacy has been reported, but its side-effects, including thrombosis, and higher relapse rate have been a problem.[7] In contrast, there is no efficacy in postoperative adjuvant therapy,[8] and the response rate currently remains 25% for advanced and recurrent cancers.[9] Recently, type 1 endometrial cancer has been on the increase, and a novel therapeutic procedure for endometrial cancer is expected. Our previous reports demonstrated that melatonin has cytostatic effects on estrogen receptor-positive Ishikawa endometrial cancer cells and its mechanism of action is mediated by receptors on the cell membrane.[10,11] In the present study, analyses of melatonin receptors related to growth inhibition in Ishikawa cells and interactions of estrogen and estrogen receptors with melatonin were investigated.

Methods

Cell line and culture

Estrogen receptor-positive Ishikawa human endometrial cancer cultured cells[12] were obtained from Professor Nozawa (Keio University). Ishikawa strains were grown as a monolayer on a 90-mm diameter dish at 37°C in an atmosphere of 5% CO_2 using 10% inactivated fetal bovine serum (FBS, Hyclone, Utah, Canada) and phenol red-free RPMI1640 (Sigma-Aldrich, Steinheim, Germany) containing 1% penicillin G/streptomycin/amphotericin B-containing antibiotic (GIBCO, NY, USA). Following confluent state, 1 mL of 0.25% trypsin-EDTA (GIBCO, Grand Island, NY, USA) was added to each dish and left at 37°C for 10 min, after which the cells were separated and collected. Thereafter, the cells were suspended in culture media and centrifuged for 5 min at 190 *g* at 4°C. Cell aggregation was suspended to become uniform after precipitation, and the number of cells was measured by hemocytometer and coulter counter, and then adjusted so that the cell count was 1×10^5/dish in a 60-mm diameter dish. 10% charcoal-treated FBS (100 mL FBS, which is supplemented with FBS containing 25 mg dextran [Sigma Chemical, St Louis, MO, USA] and 250 mg charcoal [Sigma], was centrifuged at 190 *g* for 5 min after shaken at 55°C for 45 min in a thermostatic bath, then the supernatant was collected and after repeating these procedures, it was filtered through 0.45 and 0.22 μm filters) and used for serum in culture media. These were cultured at 37°C in an atmosphere of 5% CO_2 and a variety of drugs were administered after confirmation of cell adhesion (approximately 6 h later), then used for each experiment following 96 h of culture.

Influence of luzindole and 4-phenyl-2-propionamidotetralin (4-P-PDOT) on the cytostatic effect of melatonin in Ishikawa cells

Following confirmation of cell adhesion, the cells were divided into two groups: one supplemented only with 1×10^{-9} M melatonin (WAKO, Osaka, Japan) (M group); and one supplemented with melatonin and 5×10^{-6} M luziondole (MT1/MT2 antagonist [Sigma-Aldrich, Steinheim, Germany]) or 2×10^{-6} M 4P-PDOT (MT2 selective antagonist, TOCRIS, Missouri, USA) (M+LUZ group and M+4P group, respectively); and a group supplemented with 0.1 mL of 0.005% ethanol (control group). Following 96 h of culture at 37°C, cells were collected with 0.25% trypsin after washing three times with phosphate buffered saline (PBS), after which, the viable cell count was measured. Viability of cells was determined by dye exclusion test with trypan blue (GIBCO-BRL, Grand Island, USA).

Immunocytochemistry of melatonin receptor

Cultured cells on the 35-mm diameter dish were washed three times with PBS and fixed with 4% paraformaldehyde (PFA) for 30 min. Thereafter, these cells were washed three times (5 min each time) with PBS after PFA was removed, then blocked with PBS containing 3% bovine serum albumin for 1 h. Anti-MT1 antibody and anti-MT2 antibody (both of which were anti-rabbit polyclonal antibody; MBL, Japan) were used as primary antibodies to adjust each concentration to 0.4 μg/mL for anti-MT1 and 1 μg/mL for anti-MT2, respectively. Primary antibody was allowed to react at room temperature for 12 h, then washed three times (5 min each) with PBS after the antibody solution was removed. Thereafter, 2.5 μg/mL of AP-labeled anti-rabbit IgG antibody (Invitrogen, OR, USA) was used as secondary antibody and allowed to react at room temperature for 2 h. Nucleus was stained with ProLong Gold antifade reagent with DAPI (Invitrogen) and examined by fluorescence microscopy.

Detection of melatonin receptor mRNA by polymerase chain reaction (RT-PCR)

Ishikawa cells were adjusted to 1×10^5/dish on a 60-mm diameter dish. Melatonin and 17β-estradiol

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were added after cell adhesion. The final concentrations of melatonin were changed to 0, 1×10^{-12}, 1×10^{-10}, 1×10^{-9}, 1×10^{-8}, and 1×10^{-6} M; whereas the final concentration of 17β-estradiol was set to 1×10^{-10} M. Cells were cultured at 37°C in an atmosphere of 5% CO_2 for 96 h, then total-RNA was extracted according to the acid guanidinium-phenol-chloroform (AGPC) method. DNase was used to remove genome DNA following quantitative determination with absorptiometer (SmartSpec 3000, BIO-RAD, Hercules, CA, USA). The RNA content of each sample was set to 500 μg. T3000 thermocycler (Biometra, Goettingen, Germany) and TaKaRa RNA PCR kit version 3.0 (TaKaRa, Siga, Japan) was used for RT-PCR following the protocol of the manufacturer. Expression of MT1, MT2, and ERα was examined at each melatonin level. PCR was performed using a set of primers for MT1 [5′ CCT GGT CAT CCT GTC GGT GTA TC 3′ (forward primer) and 5′ TGC TGC TGT ACA GTT TGT CGT ACT T 3′ (reverse primer)] and for MT2 [5′ CCA GCG CTG TCC GCG GT 3′ (forward primer) and 5′ CCA GAT GAG GCA GAT GTG CAG A 3′ (reverse primer)] and for ERα [5′GCA CCC TGA AGT CTC TGG AA 3′ (forward primer) and 5′ TGG CTA AAG TGG TGC ATG AT 3′ (reverse primer)] (OPERON Biotechnologies, Tokyo, Japan). PCR conditions were as follows: 30 cycles of 94°C for 30 s; 60°C for 30 s; and 72°C for 30 s. For MT1 and MT2, a second PCR was performed to detect band using one-tenth of the first PCR products under the same conditions. PCR products were electrophoresed on a 1.8% agarose gel containing ethidium bromide in the TAE buffer (Mupid 21; CosmoBio, Tokyo, Japan) for 25 min and the bands were detected with a UV transilluminator and photographed with a digital camera.

Influence of melatonin addition in anticancer drug susceptibility test

Experiments were performed using 24-well plates (International Frontier Technology Laboratory, Tokyo, Japan) containing TGP (themoreversible gelation polymer) in accordance with the protocol of the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). The details of experimental procedures have been reported[13,14] and are briefly discussed below. 300 μL of culture media was injected into each well and then the wells were allowed to stand at 37°C in an atmosphere of 5% CO_2 for 12 h. After the plates were fully cooled to 4°C, cell suspension was injected so that the number of cells were 5×10^4/well on ice, followed by 1 h of culture at 37°C in an atmosphere of 5% CO_2. 450 μL (440 μL for melatonin additive group) of culture media was added after gelatinization of medium. After 24 h of culture under the same conditions, 50 μL of each anticancer drug was added, followed by 96 h of culture under same conditions. Thereafter, 50 μg of WST-8 (2-[2-methoxyl-4-nitrophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfophenyl]-2H-tetra-zorium, monosodium salt) and 50 μL of 0.1% sodium succinate (WAKO, Tokyo, Japan) were added, then allowed to develop at 37°C in an atmosphere of 5% CO_2 for 24 h. The optical density of each plate was measured using a microplate reader (Labsystems Multiscan MS; Dainippon Pharmaceutical, Osaka, Japan) at 450 nm after each plate was cooled to 4°C for 1 hour. IC_{50} (50% inhibitory concentration) value for each drug and peak plasma concentration (PPC) of each anticancer drug was compared, and susceptibility was defined as positive when IC_{50} was lower than PPC. The following five anticancer drugs were used in the experiment: cisplatin (CDDP [Pfizer, Tokyo, Japan]), mitomysin C (MMC) (Kyowa Hakko Kogyo, Tokyo, Japan), 5-fluorouracil (5-FU [Kyowa Hakko Kogyo, Tokyo, Japan]), paclitaxel (PTX [Bristol-Myers K.K., Tokyo, Japan]) and docetaxel (DOC [Sanofi Aventis, Tokyo, Japan]). PPC of each anticancer drug was determined with 4.98 μg/mL for CDDP, 3 μg/mL for MMC, 120 μg/mL for 5-FU, 20 μg/mL for PTX, and 2.27 μg/mL for DOC. Mean IC_{50} values of the control group and melatonin additive group were compared in the experiment, and melatonin was added at the time of administration of anticancer drug and final concentration was adjusted to 1×10^{-9} M.

Statistical analysis

Analyses were conducted three times each for all experimental systems. All results were analyzed using Statcel2 (OMS Publishing, Tokyo, Japan) Stat View version 5.0 and were expressed as mean ± SE. Mann–Whitney *U*-test was used for the analysis of cell proliferation and anticancer drug susceptibility test, and statistical significance was set at $P < 0.05$.

Results

The influence of luzindole and 4P-PDOT addition on cytostatic effect of melatonin in Ishikawa cells is shown in Figure 1. The viable cell count was significantly decreased in the M group compared to the control group; however, the antiproliferative effect of melatonin disappeared even after the addition of luzindole. In contrast, the cytostatic effect of melatonin did not

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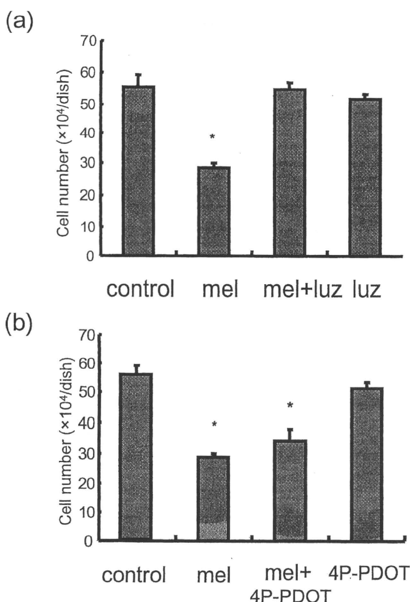

Figure 1 To monitor receptor antagonist induced cell proliferation, Ishikawa cells were incubated with 1×10^{-9} M melatonin (mel) and luzindole (luz), an MT1/MT2 antagonist (A), or 4-phenyl-2-propionamidotetraline: 4-P-PDOT(4P), an MT2 selective antagonist (B), for 96 h. Data are shown as mean ± SE. *$P < 0.05$ compared with control.

disappear due to the addition of 4P-PDOT. Thus, the cytostatic effect of melatonin is mediated by MT1 receptors, suggesting no involvement of MT2.

The immunocytochemical staining tests of MT1 and MT2 yield positive result for MT1 and negative result for MT2 (Fig. 2).

Analyses of MT1- and MT2-receptor mRNA by RT-PCR demonstrated that expression of MT1 receptor mRNA was seen, but expression of MT2 was not seen, regardless of addition of melatonin (Fig. 3a). In addition, studies looking for the effect on expression of MT1, MT2, and ERα when cultured Ishikawa cells in the presence of various concentrations of melatonin revealed that expression of MT1 receptor mRNA was not affected by melatonin level; however, expression of ERα receptor mRNA was attenuated when melatonin level was 1×10^{-9} M (Fig. 3b).

Experimental results that observed the effect of melatonin addition in the cytotoxicity reaction of a variety of anticancer drugs for Ishikawa cells are shown in Figure 4. It was determined that Ishikawa cells are susceptible to anticancer drugs tested other than DOC. In addition, the observation of influence of melatonin addition showed that a significant decrease in IC_{50} value is found in PTX, indicating enhancement of PTX action. However, enhancement of cytotoxicity reaction due to addition of melatonin was not seen in the other three anticancer drugs (CDDP, MMC and 5-FU).

Discussion

Melatonin receptor mainly exists in two subtypes: MT1 (Mel 1a); and MT2 (Mel 1b), in mammals.[15] Both are membrane-surface receptors, and *in vivo*, their expression in the suprachiasmatic nucleus, retina and cerebellum, as well as in brown fat cell, prostate, lymphocyte, small intestine and colon has been reported. In tumor cells, expression of melatonin receptors has been reported in breast cancer, ovarian cancer,[16] choriocarcinoma,[17] prostate cancer,[18] colon cancer[19] and pancreatic cancer,[20] and in particular, there have been many reports of expression in hormone-dependent tumor. However, analyses in endometrial cancer cells have been little studied. Our previous studies have revealed that melatonin has an anti-proliferative effect on ER-positive endometrial cancer cells. Its effect is inhibited by estrogen, and its mechanism of action is mediated by receptors on the cell membrane.[10,11]

Intrinsic and common actions are present for each of the actions via MT1 and MT2, and its anti-tumor effect is generally considered to be an action via MT1.[21] In Ishikawa endometrial cancer cells, although the results from analysis of receptor mRNA in the present study confirmed MT1 expression in protein and at gene level, MT2 expression was not found. We previously reported that MT2 expression may be associated with growth inhibition in Ishikawa cells,[11] and such discussion resulted from the report demonstrating that luzindole is an MT2-selective antagonist at that time of publication.[22] However, 4P-PDOT,[23] a selective antagonist of MT2, was also used to investigate cytostatic effect in this study because thereafter it was reported that luzindole is an antagonist for both MT1 and MT2, but not an MT2-selective one.[24] As a consequence, expression of MT1 rather than MT2 was seen in

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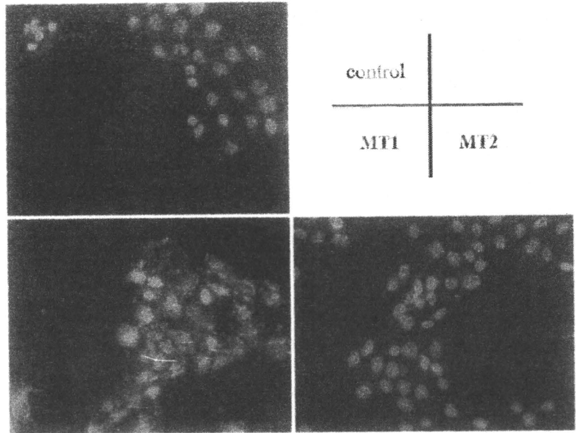

Figure 2 Immunocytochemistry of MT1 and MT2. Ishikawa cells were stained with MT1 or MT2 antibodies or phosphate buffered saline (control), followed by a second antibody labeled by AP (alkaline phosphatase). Nuclei were stained with DAPI.

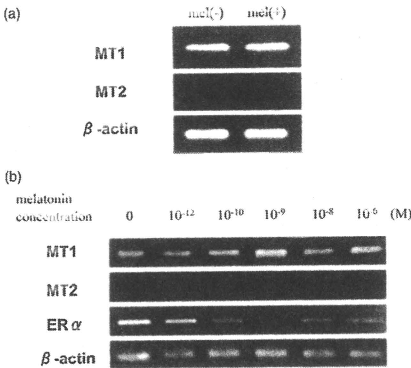

Figure 3 (A) Expression pattern of the melatonin receptor subtype mRNA on Ishikawa cells. Ishikawa cells were incubated with or without 1×10^{-9} M melatonin for 96 h. Each group has an equal amount of total RNA. (B) Expressions of MT1, MT2 and ERα mRNA on Ishikawa cells. Ishikawa cells were incubated with various concentrations of melatonin and 1×10^{-10} M 17β-estradiol for 96 h. Each group has an equal amount of total RNA.

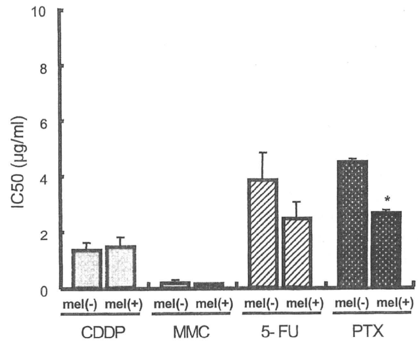

Figure 4 Effects of melatonin for anticancer drugs (cisplatin, CDDP; mitomysinC, MMC; 5-fluorouracil, 5-FU; paclitaxel, PTX; docetaxel, DOC). Ishikawa cells were incubated with or without 1×10^{-9} M melatonin for 96 h. We compared the average IC_{50} with peak plasma concentration (PPC) to determine whether or not each drug was effective on Ishikawa cells. All of the drugs except for docetaxel (DOC) were sensitive to Ishikawa cells. Data are shown as mean ± SE. $P < 0.05$ compared without melatonin (DOC are not shown).

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Ishikawa cells similar to other solid cancers, such as breast cancer, and inhibition of cell proliferation via MT1 has been confirmed. MT1 expression has also been confirmed by immunocytochemistry.

In the experiment that analyzed the influence of melatonin addition on mRNA expression in MT1, MT2 and ERα, attenuation of mRNA expression in ERα was found in the group supplemented with 1×10^{-9} M melatonin. These observations suggested that ERα expression in Ishikawa cells is inhibited in the presence of melatonin concentration at the physiological level. Molis *et al.* demonstrated that cell proliferation is inhibited optimally by melatonin addition with concentration at the physiological level in breast cancer cell line, MCF-7, and that its mechanism is an attenuation of mRNA expression level in ERα.[25] Furthermore, it was suggested that the steady state of ER mRNA level is mainly determined by transcriptional regulation of genes, and only melatonin at physiological concentration provides its control, which is not provided by other concentrations.[25] Kiefer *et al.* reported that melatonin at physiological concentration attenuates the ER-dependent transcriptional activity of ER genes in MCF-7.[26] These results suggest that melatonin may indirectly inhibit the proliferative action of E2 on Ishikawa cells by attenuation of ERα expression in Ishikawa endometrial cancer cells. However, our previous studies confirmed that melatonin also inhibits proliferation of Ishikawa cells in a culture condition in the absence of E2;[10] therefore, all mechanisms of action of growth inhibitory effect on endometrial cancer cell could not be explained. Mechanisms of action, including the effect on other growth factors other than E2 and cell cycle, is also suggested.[27] Further study is warranted to analyze these mechanisms.

Recently, the usefulness of concomitant use of melatonin and chemotherapy[28] (i.e. biochemotherapy) has been reported. Although postoperative chemotherapy for endometrial cancer and chemotherapy for the advanced/recurrent cases are still less than well-established, potential candidates include taxan-based antineoplastic drugs.[29] Although the mechanism is unclear, our *in vitro* study demonstrated that melatonin could enhance the cytocidal effect of paclitaxel, suggesting a potential use of melatonin for biochemotherapy of endometrial cancer. The antitumor effect of melatonin is based on mechanisms such as (i) direct induction of apoptosis in tumor cells and inhibition of apoptosis in normal cells; (ii) activation of antitumor immune system; and (iii) regulation of oncogene.[30] Lissoni has reported that cases with complete response plus partial response were significantly increased in a group receiving chemotherapy plus oral melatonin compared with chemotherapy alone for patients with a variety of solid cancer, suggesting the results were due to oncostatic, immunomodulating, and antioxidant properties of melatonin.[28] Based on our results, it is necessary to prove the role of melatonin for biochemotherapy and biochronotherapy through further *in vitro* studies.

Inhibition of E2-dependent tumor cell proliferation by melatonin includes the pathway that influences tumor cells directly via receptors and the pathway that inhibits E2 secretion from local ovaries.[3] The former is thought to involve the mechanism that inhibits the action of aromatase, which generates E2 from androgen secreted by the adrenal gland (SEEM: selective estrogen enzyme modulator) and the mechanism that inhibits the action and expression of ER in the nucleus (SERM: selective estrogen receptor modulator). *In vivo* experiments in an animal model and *in vitro* experiments in a breast cancer cell line supported the hypothesis that the action of melatonin is mainly due to the former.[3] Because ovarian estrogen secretion diminishes after menopause, the hypothesis supports the premise that the action of melatonin can be expected even after menopause. Melatonin tends to decrease after peaking in puberty;[31] thus, sufficient secretion of melatonin may not be expected at the perimenopausal age.

Our study revealed that melatonin inhibits the expression of ERα in Ishikawa endometrial cancer cultured cells and that melatonin may enhance the antitumor effect by chemotherapy, especially in combination with paclitaxel. Alonso *et al.* demonstrated that direct exposure to E2 reduces melatonin synthesis and release in female rat pinealocytes.[32] Moreover, a recent study showed that women who work rotating night shifts for a long duration have a significant increased risk of endometrial cancer,[33] which suggests that insufficient secretion of melatonin as a result of night work might be a risk in endometrial cancer. Thus, administration of melatonin may prevent the onset of endometrial cancer in early postmenopausal women or women working at night who have decreasing melatonin. In the future, melatonin should be investigated as a possible biochemotherapeutic modulator in endometrial cancer.

Acknowledgment

The authors are grateful to Dr Tomoya Akama, Dr Tatsuru Ohara and Mr Wataru Tarumi for their technical assistance.

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Inhibitory effect of rice bran-derived crude glycosphingolipid on colon preneoplastic biomarker lesions induced by azoxymethane in male F344 rats

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Received September 2, 2008; Accepted October 9, 2008

DOI: 10.3892/mmr_00000060

Abstract. The aim of the present study was to examine whether crude glycosphingolipid (cGSL) has short-term chemopreventive effects on the preneoplastic biomarker lesions involved in carcinogen-induced rat colon carcinogenesis. We also examined whether cGSL affects cell proliferation and apoptosis in these lesions. The crude preparation was obtained by the simple ethanol extraction method. Five-week-old male F344 rats were divided into 6 groups. Rats in groups 1-4 were given subcutaneous injections of azoxymethane (AOM) (20 mg/kg body weight) once a week for 2 weeks. Starting 1 week before the first injection of AOM, the rats in groups 2, 3 and 4 were fed a diet containing 250, 1,000 and 3,000 ppm cGSL, respectively, for 5 weeks. The experiment was terminated 5 weeks after the start date, and the number of aberrant crypt foci (ACF) and mucin-depleted foci (MDF) was counted. Dietary cGSL significantly inhibited the induction of ACF (group 3, $P<0.01$; group 4, $P<0.05$) and MDF (groups 2 and 3, $P<0.001$; group 4, $P<0.05$) as compared to group 1 treated with AOM alone. In groups 3 and 4, proliferating cell nuclear antigen-positive indices of epithelial cells were significantly lower than in group 1 (group 3, $P<0.05$; group 4, $P<0.005$). Caspase-3-positive indices were significantly higher in groups 3 and 4 than in group 1 (group 3, $P<0.01$; group 4, $P<0.001$). These results suggest that dietary cGSL had a potent chemopreventive effect in the present short-term colon carcinogenesis bioassays, and that this effect may be associated with the inhibition of ACF and MDF and the induction of apoptosis.

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Key words: glycosphingolipid, rice bran, prevention, preneoplastic lesions, colon

Introduction

Sphingolipids are a group of structural and bioactive derivatives that have a long chain backbone called a sphingoid base (sphingosine). These compounds have been regarded as inert structural components of cell membranes. However, in recent years sphingolipids have emerged as an important group of signaling molecules involved in cellular events such as immunoresponse, differentiation, cell proliferation and apoptosis. It was demonstrated that ceramide causes growth inhibition and induces morphological differentiation in HL-60 human myelocytic leukemia and neuroblastoma cell lines (1,2). Obeid *et al* reported that tumor necrosis factor-α stimulates the production of ceramide and the induction of apoptosis by the hydrolysis of sphingomyelin (3). Other reports have indicated that the proapoptotic effect of ceramide is induced by the activation of Fas or radiation (4,5). As a result, sphingolipids are attracting attention as a promising target of cancer chemoprevention and therapy.

Aberrant crypt foci (ACF) were proposed as colon preneoplastic lesions by Bird (6,7) and are widely used as reliable biomarkers of colon carcinogenesis (8). Mucin-depleted foci (MDF) are lesions observed as mucin defects in rat colonic mucosa stained with high iron diamin-Alcian blue, and their histological characteristics demonstrate dysplastic features (9). Additionally, MDF are significantly correlated with the development of colon tumors in azoxymethane (AOM)- or 1,2-dimethylhydrazine (DMH)-treated rats (9-11). Furthermore, both ACF and MDF are valuable biomarkers for short-term colon carcinogenesis bioassays, and are useful for the examination of possible chemopreventive effects of a wide variety of candidate agents (12,13).

Complex sphingolipids such as glycosphingolipid and sphingomyelin display biological activities similar to those of ceramide. Recent studies have shown that dietary intake of synthesized or naturally-occurring sphingomyelin and glycosphingolipids prevents colon carcinogenesis in DMH-treated or multiple intestinal polyposis (Min) mice (14-18).

Sphingolipids can be found in milk, eggs and soybeans; foods that are commonly consumed in everyday life as a dietary supplement. Although sphingolipids exhibit a variety

of biological activities, the precise mechanisms by which the complex causes tumor suppression are not known.

In the present study, we examined whether crude glycosphingolipid (cGSL) obtained from rice bran actually inhibits the development of carcinogen-induced preneoplastic lesions such as ACF and MDF in the rat colon. Furthermore, to investigate its mechanisms of action on cell proliferation and apoptosis, immunohistochemical analysis of proliferating nuclear cell antigen (PCNA) and cleaved caspase-3 was performed.

Materials and methods

Animals, diets and chemicals. Four-week-old male F344 rats were purchased from Japan SLC Inc. (Hamamatsu, Japan). All animals were housed in wire cages (3 rats/cage) with free access to drinking water and the control diet (CE-2, Clea Japan Inc., Tokyo, Japan) under controlled humidity (50±10%), lighting (12-h light/dark cycle) and temperature (23±2˚C) conditions. Composition of the CE-2 diet was water (8.6%), protein (24.9%), fat (4.6%), fiber (3.7%), ash (6.7%) and nitrogen-free extract (51.4%). cGSL was provided by Oryza Oil & Chemical Co. Ltd. (Owari-Ichinomiya, Japan), and was extracted from rice bran by the ethanol extraction method. High performance liquid chromatography (HPLC) analysis revealed that the cGSL contained monoglucosylceramide (8.6%), sterol (5.0%), triglyceride and diglyceride (26.4%), calcium carbonate (53.0%) and starch (7.0%).

Experimental design. Animal experiments were approved by the Animal Welfare Committee of the University of the Ryukyus. After being quarantined for 1 week, 42 male F344 rats were divided into 6 groups. Starting at 5 weeks of age, the rats in groups 1 to 4 (n=9/group) received AOM (Nard Institute Ltd., Hyogo, Japan, 20 mg/kg body weight, s.c. injection) once a week for 2 weeks. Rats in groups 2, 3 and 4 were fed a diet containing 250, 1,000 and 3,000 ppm cGSL, respectively, throughout the experiment. The concentration of cGSL was determined according to our previous study (19). Rats in group 5 (n=3) were fed a diet containing 3,000 ppm cGSL to examine the adverse side effects of the complex. Rats in group 6 (n=3) were fed the control diet alone and served as negative controls. At 5 weeks from the start date, all animals were euthanized under CO_2 anesthesia. Colon tissues were removed, washed with saline, opened longitudinally and fixed with 10% buffered formalin.

Detection of aberrant crypt and mucin-depleted foci. The staining procedure for ACF and MDF was performed as described in our previous study (11). The fixed colon tissues were stained in an Alcian blue solution (Sigma Chemical Co., St. Louis, MO) for 5 min and immediately washed with distilled water, then placed on a glass plate with the mucosal side up. Using a light microscope at a magnification of x40, ACF and MDF were counted. Briefly, ACF were identified according to the following criteria: larger than and elevated above the adjacent normal crypts, with thickened cell walls lining the crypt and increased pericryptal area (11). MDF were identified as focal lesions characterized by the absence or very small production of mucins (11).

Immunohistochemical analysis. After ACF and MDF counting, colon tissues were rolled up, embedded in a Swiss roll form and subjected to immunohistochemical staining of PCNA and cleaved caspase-3. Immunohistochemical staining of PCNA is widely applied to examine cell proliferation (20). Caspase-3 is a pivotal apoptotic protein. Its active form, cleaved caspase-3, is generated by the proteolysis of its zymogen and exists only in the activated apoptotic cascade. Therefore, the detection of cleaved caspase-3 is a sensitive indicator of apoptosis (21). Sections (4-μm) were deparaffinized and rehydrated, then incubated in 3% H_2O_2 for 20 min in order to block endogenous peroxidase activity. Sections were boiled in 1 mM EDTA (pH 8.0) for 10 min in a pressure cooker. Subsequently, the sections were incubated with primary antibodies of PCNA (1:100 dilution, Dako Co. Ltd., Kyoto, Japan) or cleaved caspase-3 (1:200 dilution, Cell Signaling Technology Inc., Beverly, MA) at room temperature for 60 min, then secondary antibody treatment was performed using Histofine Simple Stain (Nichirei, Tokyo, Japan). Visualization of immunoreactivity was performed with the Liquid DAB Substrate Chromogen System (Dako Co., Copenhagen, Denmark). To determine the PCNA-positive (PCNA PI) and caspase-3-positive indices (caspase-3 PI), 15 visible crypts per colon were chosen, and at least 1,500 epithelial cells were counted. Positive indices were calculated as the percentage of positive cells with respect to the total number of cells counted.

Statistical analysis. One-way ANOVA was performed to analyze the data, and statistical differences were determined by Dunnet's test. All statements of significance are $P<0.05$.

Results

General observations. A total of 42 rats survived to the end of the experiment, and none developed colon tumors. To ascertain whether the dietary administrtion of cGSL caused any toxic adverse side effects on body weight gain, the rats were monitored on a weekly basis. AOM and/or cGSL were not observed to have any significant effects on body, liver or kidney weight (data not shown). Dietary cGSL at 250, 1,000 and 3,000 ppm caused no symptomatic side effects in any of the rats.

Inhibition by cGSL of the occurrence of aberrant crypt and mucin-depleted foci. All rats in groups 1-4 developed ACF and MDF in their colonic mucosa. No ACF or MDF were observed in any of the rats in groups 5 and 6. The total number of ACF was significantly lower in AOM-treated rats fed a 1,000 and 3,000 ppm cGSL diet than in the control rats (1,000 ppm, $P<0.01$; 3,000 ppm, $P<0.05$; Table I), and the total number of MDF was significantly lower in AOM-treated rats fed a cGSL diet than in the control rats (250 ppm, $P<0.001$; 1,000 ppm, $P<0.001$; 3,000 ppm, $P<0.05$; Table I). The number of ACF or MDF that contained >4 crypts was not modified by the treatments. These results indicate that dietary cGSL inhibits the occurrence of ACF and MDF induced by AOM.

Inhibition of proliferating cell nuclear antigen-positive index by cGSL on the colonic epithelial cells. To determine whether cGSL affects cell proliferation in carcinogen-treated colonic

Table I. Inhibition of the occurrence of aberrant crypt and mucin-depleted foci by crude glycosphingolipid.

Group no.	Treatment	Total no. of ACF	No. of ACF containing >4 ACs	Total no. of MDF	No. of MDF containing >4 ACs
1	AOM	138.2±24.8[a]	13.8±4.9	13.9±4.1	0.6±0.7
2	AOM+250 ppm cGSL	116.1±16.8	13.4±4.3	8.2±1.9[b]	0.3±0.5
3	AOM+1,000 ppm cGSL	97.9±17.7[c]	10.7±4.3	7.8±2.5[b]	0.4±0.5
4	AOM+3,000 ppm cGSL	105.9±37.6[d]	10.8±5.0	9.8±2.4[d]	0.3±0.7
5	3,000 ppm cGSL	0	0	0	0
6	None	0	0	0	0

[a]Mean ± SD. Significantly different from group 1: [b]$P<0.001$, [c]$P<0.01$, [d]$P<0.05$; Dunnet's test. cGSL, crude glycosphingolipid; ACF, aberrant crypt foci; MDF, mucin-depleted foci; ACs, aberrant crypts; AOM, azoxymethane.

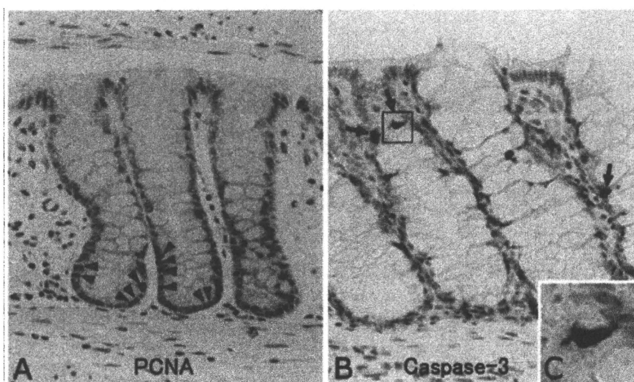

Figure 1. Representative results of immunohistochemical staining of PCNA (A) and caspase-3 (B and C) in the rat colonic epithelium. PCNA immunoreactivity is positive in the nucleus (arrow heads). Caspase-3-positive cells are mainly localized in the upper half of the crypt (arrows), and immunoreactivity shows the cytoplasmic pattern at high power magnification (C).

Table II. Inhibition of the proliferating nuclear cell antigen-positive index by crude glycosphingolipid in colonic epithelial cells.

Group no.	Treatment	PCNA PI
1	AOM	5.28±1.18[a]
2	AOM+250 ppm cGSL	3.99±0.74
3	AOM+1,000 ppm cGSL	3.92±0.35[b]
4	AOM+3,000 ppm cGSL	3.47±0.42[c]
5	3,000 ppm cGSL	3.29±0.50
6	None	3.25±0.34

[a]Mean ± SD. Significantly different from group 1: [b]$P<0.05$, [c]$P<0.005$; Dunnet's test. PCNA PI, proliferating nuclear cell antigen-positive index; cGSL, crude glycosphingolipid; AOM, azoxymethane.

mucosa, we measured the PCNA PI in epithelial cells by immunohistochemistry (Fig. 1). As shown in Table II, treatment of rats with 1,000 and 3,000 ppm cGSL caused a significant decrease in the PCNA PI (1,000 ppm, $P<0.05$; 3,000 ppm, $P<0.005$) as compared to the control group (group 1). This inhibition occurred in a dose-dependent manner. The PCNA PIs of groups 1 and 6 were 5.28 and 3.25, respectively. In groups 2, 3 and 4, the PCNA PI was 3.99, 3.92 and 3.47 in AOM-treated rats fed 250, 1,000 and 3,000 ppm cGSL, respectively. These findings suggest that dietary cGSL normalizes the increase in cell proliferation induced by AOM.

Effect of cGSL on apoptosis. Since cGSL is able to normalize increases in cell proliferation in carcinogen-treated colonic epithelial cells, we examined whether this inhibition by cGSL is due to the induction of apoptosis. After the injection of AOM, treatment of rats with 1,000 and 3,000 ppm cGSL

Table III. Inhibition of the caspase-3-positive index by crude glycosphingolipid in colonic epithelial cells.

Group no.	Treatment	Caspase-3 PI
1	AOM	0.55±0.67[a]
2	AOM+250 ppm cGSL	0.62±0.16
3	AOM+1,000 ppm cGSL	0.85±0.23[b]
4	AOM+3,000 ppm cGSL	0.94±0.20[c]
5	3,000 ppm cGSL	1.01±0.13
6	None	0.97±0.04

[a]Mean ± SD. Significantly different from group 1: [b]P<0.01, [c]P<0.001; Dunnet's test. PI, positive index; cGSL, crude glycosphingolipid; AOM, azoxymethane.

caused a significant increase in the caspase-3 PI as compared to the control group (group 1) (1,000 ppm, P<0.01; 3,000 ppm, P<0.001; Table III). This increase occurred in a dose-dependent manner. These results indicate that cGSL induces apoptosis in colonic epithelial cells treated with AOM.

Discussion

Complex sphingolipids display a wide spectrum of biological activities including cell proliferation, differentiation and apoptosis. However, the precise mechanisms by which this complex exerts a cancer preventive effect are unknown. This study provides the first detailed examination of the inhibitory effects of rice bran-derived cGSL, which was extracted with safe solvents only, on 2 different categories of carcinogen-induced rat colon preneoplastic lesions, ACF and MDF. In the present study, we found that dietary administration of cGSL significantly inhibited the occurrence of ACF and MDF induced by the carcinogen AOM. We also found that the PCNA PI in colonic epithelial cells was inhibited in a dose-dependent manner by the treatment of rats with cGSL. Furthermore, dietary cGSL increased the caspase-3 PI in AOM-treated colonic epithelial cells. These findings suggest that cGSL prevents colon preneoplastic lesions, and that this effect may be associated with the inhibition of cell proliferation and the induction of apoptosis. Similar results have been reported in previous studies, which demonstrated that dietary intake of synthesized ceramide and milk-derived sphingolipids inhibited colonic cell proliferation in DMH-treated or Min mice (18,22). As well, sphingosine, sphinganine and C_2-ceramide induced apoptosis in HT29 and HCT116 human colon carcinoma cell lines (23). These findings, together with the results of the current study, suggest that cGSL has cancer preventive potential in a short-term colon carcinogenesis bioassay.

In the present study, the suppressive effect of cGSL on the occurrence of ACF and MDF was not statistically significant at a lower dose (250 ppm). At higher doses (1,000 and 3,000 ppm), the inhibitory effect of cGSL on the occurrence of ACF and MDF was not dose-dependent. It is possible that a dose level of 250 ppm cGSL was not sufficient to suppress the formation of these lesions, while at 1,000 ppm cGSL had already reached its maximum effect. It seems likely that had we used a larger number of animals, and had the experiment continued for a longer period, the number of ACF or MDF would have decreased further in the high-dose cGSL-treated groups.

Ceramide, sphingosine and sphinganine are considered to play a pivotal role in tumor suppression (24,25). Schmelz *et al* have suggested that complex sphingolipids in food are hydrolyzed to ceramide in the intestinal tract and taken up by colonic cells, thus performing its biological activity (16,17). Most sphingolipids are hydrophobic, and are thus insoluble in aqueous solution. There have been no studies investigating the colon cancer preventive effect of cGSL, and no epidemiological data are available regarding the relation between the daily consumption of sphingoid-rich food (e.g., rice bran, milk, eggs and soybeans) and colon cancer risk. Therefore, to clarify whether its effect is confined to colon cancer requires further investigation. The ceramide-mediated signaling pathways involved in tumor suppression are extensive (24,25). The induction of apoptosis and the inhibition of cell proliferation are considered to be key events in tumor suppression (23,26). Molecules such as RB, Bcl-2, p53, mitogen-activated protein kinases and protein kinase C are the downstream targets of ceramide (24,25). Ceramide activates Bcl-2, and this proapoptotic protein eventually activates caspase family molecules through the release of cytochrome c from mitochondria (27,28). Schmelz *et al* demonstrated that sphingolipids normalize the localization of the ß-catenin protein in the colonic mucosa (29). Aberrant expression of the ß-catenin protein has been demonstrated in carcinogen-induced MDF in rats (10,11). We found that the development of MDF was inhibited by cGSL treatment, suggesting that the alteration of the signaling pathway via ß-catenin may play an important role in the modulation of carcinogen-induced colon carcinogenesis. However, this aspect is in need of further study.

In summary, we demonstrated the inhibitory effect of dietary cGSL on 2 different biomarker lesions, ACF and MDF, in azoxymethane-treated F344 rats. The conceivable mechanism of action of cGSL might be the inhibition of cell proliferation and the induction of apoptosis in the colonic mucosa. With these considerations, cGSL may have had a potent chemopreventive effect in our short-term colon carcinogenesis bioassay system. Further studies are in progress to determine whether dietary cGSL suppresses tumor formation in long-term experiments.

Acknowledgements

This work was supported, in part, by a Grant-in-Aid from the Ministry of Health, Labor and Welfare and a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan. We thank Tomoko Sugishita and Koji Kanazawa (Oryza Oil & Fat Chemical Co. Ltd.) for supplying cGSL.

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Journal of Obstetrics and Gynaecology, August 2008; 28(6): 634–637

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Heparanase expression in endometrial cancer: Analysis of immunohistochemistry

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Summary

The human enzyme heparanase has been shown to function in tumour progression, metastatic spread and tumour angiogenesis. The aim of the present study was to assess heparanase expression assessed by immunohistochemical staining (IHC) in endometrial cancer in correlation with clinicopathological factors. A total of 52 endometrial cancers were obtained from previously untreated patients (median age, 56 years, range, 35–80 years). The expression of heparanase was evaluated by using IHC with anti-heparanase polyclonal antibody. This antibody was raised by immunising a rabbit with a peptide containing the amino acid residues from 238 to 250 of the heparanase. The IHC data were used to determine the relationship between heparanase expression, and clinicopathological parameters. IHC showed that the heparanase was expressed in 23 of 52 (44.2%) endometrial cancers. Heparanase was abundantly and equally expressed in both the cytoplasm and the cell membrane of the cells in endometrial cancer. Strong heparanase-positive staining was also seen at the invasive front of the tumour into myometrium. The expression was significantly related to lymph-vascular space involvement ($p = 0.0028$), depth of myometrial invasion ($p = 0.0026$), and histological tumour grade ($p = 0.0135$). In six tumours with positive lymph nodes, the heparanase expression was observed as being higher compared with tumours with negative lymph nodes, which was not significant ($p = 0.2349$). In terms of peritoneal cytology, ovarian metastasis, and cervical invasion, we observed no significant difference in the heparanase expression assessed by IHC. These results suggest that the expression of heparanase may promote tumour invasion into myometrium and lymph vascular space in endometrial cancer.

Keywords

Endometrial cancer, heparanase, immunohistochemistry, polyclonal anti-heparanase antibody, tumour invasion

Introduction

The human enzyme heparanase, an endo-beta-glucuronidase that cleaves heparan sulfate (HS) at specific intrachain sites, has been shown to function in tumour progression and metastatic spread. (Vlodavsky et al. 1999; Hulett et al. 1999) Degradation of heparan sulfate proteoglycans by heparanase appears to play an important role in the invasiveness of tumour cells through the basement membrane and into the extracellular matrix. Tumour spread involves degradation of various components of the extracellular matrix and blood vessel wall. Among these is heparan sulfate proteoglycan, which plays a key role in the self-assembly, insolubility and barrier properties of basement membranes and extracellular matrices (Marchetti et al. 2000; Ilan et al. 2006). Expression of heparanase, which degrades heparan sulfate correlates with the metastatic potential of tumour cells, and treatment with heparanase inhibitors markedly reduces the incidence of metastasis in experimental animals (Vlodavsky et al. 1999; Nakajima et al. 1984; Toyoshima and Nakajima 1999). Heparanase may thus facilitate both tumour cell invasion and neovascularisation, two critical steps in tumour progression.

A majority of patients with endometrial cancer have a 5-year survival rate of approximately 90%. However, 15–25% of patients with a tumour extending outside the uterus but limited to the true pelvis (FIGO stage III), have an estimated 5-year survival of 40–70% (Wolfson et al. 1992; Greven et al. 1993). Also, in patients with stage III disease, lymph vascular space involvement, deep myometrial invasion and lymph node metastasis are reported to be independent prognostic factors (Aoki et al. 2001, 2004). It is quite interesting to investigate the relationship between heparanase expression and tumour aggressiveness in association with the prognostic variables in endometrial cancer.

Materials and methods

Tissue samples

A total of 52 endometrial cancers were obtained from previously untreated patients (median age, 56 years, range, 35–80). Tissues obtained at laparotomy were formalin-fixed and paraffin-embedded for standard H&E staining and immunohistochemical study. Informed consent for using tumour tissues obtained for scientific research in the future was obtained from the patients. All cases underwent

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ISSN 0144-3615 print/ISSN 1364-6893 online © 2008 Informa Healthcare USA, Inc.
DOI: 10.1080/01443610802323542