with a peptide containing the amino acid residues from 238 to 250 of the Heparanase. Tumor angiogenesis was assessed using microvessel counting. The Mann-Whitney U test, one factor ANOVA test, and Spearman's test were used to determine the relationship between heparanase expression, microvessel density, and clinicopathological parameters.

***Results:*** The expression of heparanase mRNA was detected in 26 of 52 (50%) endometrial cancers, and was significantly correlated with FIGO stage IIIc (p=0.0075), the presence of lymph-vascular space involvement (LVSI) (p=0.0041), lymph node metastasis (LNM) (p=0.0049), and histological tumor grade (p=0.003). IHC showed that the heparanase was expressed in 23 of 52 (44.2%) endometrial cancers, which was significantly related to LVSI (p=0.0028), depth of myometrial invasion (p=0.0026), and histological tumor grade (p=0.0135). Microvessel density was also associated with FIGO stage IIIc (p=0.027), LVSI (p=0.001), LNM (p=0.038), ovarian metastasis (p=0.03) and histological tumor grade (p=0.003). Moreover, we found a strong positive correlation between heparanase expression and microvessel density ($r^2$=0.475, p=0.0001).

***Conclusion:*** These results suggest that the expression of heparanase can promote tumor angiogenesis and develop metastasis in endometrial cancer.

## Introduction

The human heparanase gene, an endo-beta-glucuronidase that cleaves heparan sulfate (HS) at specific intrachain sites, has recently been cloned and has been shown to function in tumor progression and metastatic spread. Degradation of heparan sulfate proteoglycans by heparanase appears to play an important role in the invasiveness of tumor cells through the basement membrane and into the extracellular matrix. Recent cloning of the heparanase gene and generation of monoclonal antibodies against the enzyme allow us to examine the tumor cell expression of the enzyme [1, 2]. Heparanase expression is well correlated with the metastatic cancer potential of several different types of cancer. It has been reported that heparanase is strongly expressed in highly metastatic cells, such as melanoma [3], gastric cancer [4], bladder cancer [5], pancreas cancer [6, 7], hematogeneous [8], oral carcinoma [9], hepatocellular carcinoma [10], and colon cancer [11].

Heparan sulfate proteoglycans also bind and sequester a variety of bioactive proteins, including growth factors, chemokines, cytokines, and enzymes [12, 13]. Heparanase produced by a given tumor may facilitate tumor invasion and angiogenesis through the release of HS-

bound growth factors, such as basic fibroblast growth factor (bFGF) [14, 15], vessel endothelial growth factor (VEGF) [16], hepatocyte growth factor (HGF) [17], and platelet-derived growth factor (PDGF) [18]. Thus, expression of heparanase is thought to play an important role in cancer invasion and metastasis [19-21].

A majority of patients with endometrial cancer are diagnosed as being without clinical evidence of extrauterine spread (the International Federation of Gynecology and Obstetrics (FIGO) stage I and II) and have a 5-year survival rate of approximately 90%. However, 15-25% of patients with a tumor extending outside the uterus but limited to the true pelvis (FIGO stage III) and have an estimated 5-year survival of 40 to 70% [22, 23]. 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 [24-26]. It is quite interesting to investigate the relationship between heparanase expression and angiogenesis in association with the prognostic variables in endometrial cancer. Here we review our data on heparanase expression and angiogenesis in endometrial cancer [27-29].

## Materials and Methods

### *Tissue Samples*

A total of 52 endometrial cancers were obtained from previously untreated patients (median age, 56 years, range, 35-80). Tissue samples were snap-frozen in liquid nitrogen and maintained at -80°C until use. All cases underwent curative operations for endometrial cancer and were surgically categorized into different stages according to FIGO staging.

### *RNA extraction and cDNA synthesis*

Total cellular RNA was extracted from tissues using Isogen (Nippon Gene) according to the manufacturer's specific protocol. First-strand cDNA was synthesized from total cellular RNA primed by random hexamers. Briefly, 1 μg of total cellular RNA was incubated in 20 μl reactions containing 200 U of Moloney murine leukemia virus reverse transcriptase (BRL), 50 mM Tris-HCl (pH 8.3), 75 mM potassium chloride, 3 mM magnesium chloride, 10 mM DTT, 100 mg/ml BSA, 0.625 mM each of four dNTPs (Pharmacia), 0.4 μg of random hexamers (Pharmacia), and 20 U of RNasin (Promega) for 45 min at 37°C. The reaction was terminated by boiling for 5 min.

### *Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis*

PCR primers were designed from the cDNA sequences of the heparanase [1]. The sequence of the nucleotides used for heparanase was: forward 5'-TTC GAT CCC AAG AAG GAA TCA AC-3' and reverse 5'-GTA GTG ATG CCA TGT AAC TGA ATC-3'. The primers used for β-actin were as follows; forward: 5'-GAT GAT GAT ATC GCC GCG CT-3' and reverse: 5'-TGG GTC ATC TTC TCG CGG TT-3'. Two μl of the cDNA reaction was amplified in a volume of 100 μl containing 50 mM potassium chloride, 10 mM Tris-HCl (pH 9.0), 1.5 mM magnesium chloride, 0.1% Triton X-100, 100 mM each of four dNTPs (Pharmacia), 250 nM of a pair of primers, one unit of Taq polymerase (Takara). The amplification reaction consisted of 33 cycles, including denaturation at 94°C for 1 min, annealing for 1 min at 60°C and extension at 72°C for 1.5 min. At the end of the reaction a polymerization step at 72°C for 7 min was included. PCR products were run on a 1.2% agarose gel and visualized by ethidium bromide (Sigma). Gel images were obtained using FAS-II UV-image analyzer (Toyobo), and the densities of the products were quantified using the Quantity One eversion 3.0 (PDL, Inc.). The relative expression levels were calculated as the density of the product of the respective target genes divided by that of the β-actin from the same cDNA.

### *Immunohistochemistry (IHC)*

Tissue sections were dewaxed with xylene and rehydrated in alcohol. Slides were then heat inactivated in 10 mmol/L sodium citrate (pH 6.0) in a microwave for 3 minutes. Cooled slides were rinsed with PBS and then incubated with 1% $H_2O_2$ in methanol for 30 minutes at room temperature. Sections were then blocked with 5% normal goat serum in PBS for 30 minutes at room temperature followed by 1 hour incubation with an antiheparanase rabbit serum (1:500 dilution) in PBS. This antibody was raised by immunizing a rabbit with a peptide containing the amino acid residues from 238 to 250 the 50-kDa HPR1 subunit and is able to detect both the 50-kDa and 65-kDa forms of expressed HPR1. Specificity of this antibody was well characterized by Western blot. Slides were washed and then incubated with goat antirabbit IgG-biotin conjugate (PharMingen) diluted at 1:300 in 5% human serum in PBS. Streptavidin-heparanase conjugate (Zymed Laboratories) diluted at 1:200 in PBS with 5% normal human serum was added and incubated for 45 minutes at room temperature. Color was developed by DAB substrate (Sigma) followed by 3,3 =diaminobenzidine enhancer (Vector Laboratories). Slides were counterstained with Mayer's hematoxylin for 2 minutes, dehydrated, and mounted. Heparanase expression was determined by a board-certified pathologist and corroborated

independently by three investigators in this study, all blinded to other clinicopathologic information. Heparanase expression was judged as positive by the presence of brown staining, specifically within 20% of the tumor areas. In the majority of specimens, heparanase staining was present in all tumor cells. A graded scoring system was not used because of the variation in intensity of heparanase signal between the experiments conducted at different times.

### *Evaluation of microvessel density (MVD)*

Microvessel counting was used for assessing tumor angiogenesis. Three areas of high vascular density (hot spot) were selected at low power (x40 and x100). Counts were performed with a microscope at x250 magnification, corresponding to 0.384 $mm^2$ field size, and the average value of these three areas was taken as the microvessel score. Only blood vessels with a well-defined lumen or a linear vessel shape were taken into account.

### *Statistical analysis*

A one-factor ANOVA test, and a Mann-Whitney U test were used to determine the relationship between the heparanase expression, or microvessel density, and clinicopathological factors. Associations between heparanase expression and the clinicopathologic parameters of endometrial cancer specimens were determined by chi-square or Fisher's exact test. The correlation between heparanase mRNA expression and microvessel density was assessed using a Spearman's test. P values less than 0.05 were considered statistically significant for all tests.

## Results

### *Heparanase mRNA expression and clinicopathological factors*

Heparanase expression was detected in 26 of 52 (50%) endometrial cancer tumors using RT-PCR. Figure 1 demonstrates the final RT-PCR products of heparanase and β-actin on a 1.2 % agarose gel. The ratios of heparanase/β-actin ranged from 0 to 0.933 with a mean value of 0.062±0.284. The ratios of heparanase/β-actin in each clnicopathological factors are presented in Table 1. FIGO stage IIIc tumors expressed significantly higher heparanase mRNA compared with stage I (p= 0.0005) or IIIa (p=0.0126) tumors. Grade 2 and 3 tumors showed a higher heparanase expression than those of grade 1 tumors, significantly (0.425±0.334 vs. 0.137±0.213, p=0.0033). In respects to LVSI tumors with positive LVSI had a significantly stronger expression of heparanase than those with negative LVSI (0.361±0.262 vs. 0.157±0.274,

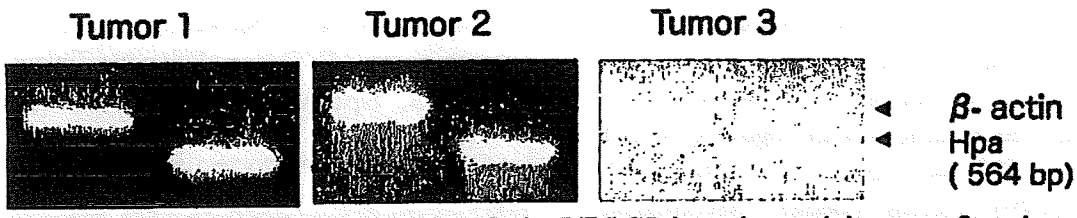


**Figure 1:** Detection of heparanase mRNA by RT-PCR in endometrial cancer; β-actin was used as a positive control.

**Table 1:** Correlation between heparanase expression by RT-PCR and clinicopathological factors

| Variables | No. of patients | Mean±S.D.* | P-value* |
|---|---|---|---|
| FIGO stage | | | $p$=0.0075 |
| IA | 6 | 0 | p=0.0005 |
| IB | 27 | 0.228±0.304 | p=0.0097 |
| IC | 7 | 0.116±0.114 | p=0.061 |
| II | 1 | 0.237 | p=0.0126 |
| IIIa | 4 | 0.193±0.145 | |
| IIIc | 7 | 0.558±0.357 | |
| Tumor grade | | | |
| 1 | 40 | 0.137±0.213 | $p$=0.0033 |
| 2+3 | 12 | 0.425±0.334 | |
| Myometrial invasion | | | NS |
| a+b | 36 | 0.080±0.158 | |
| c | 16 | 0.284±0.360 | |
| LVSI* | | | $p$=0.0041 |
| Yes | 18 | 0.361±0.262 | |
| No | 34 | 0.157±0.274 | |
| Lymph node metastasis | | | $p$=0.0049 |
| Yes | 6 | 0.526±0.336 | |
| No | 46 | 0.159±0.230 | |
| Peritoneal cytology | | | NS |
| Yes | 2 | 0.322±0.248 | |
| No | 50 | 0.206±0.289 | |
| Ovarian metastasis | | | NS |
| Yes | 4 | 0.131±0.162 | |
| No | 48 | 0.234±0.294 | |
| Cervical invasion | | | NS |
| Yes | 5 | 0.343±0.081 | |
| No | 47 | 0.210±0.296 | |

* LVSI, lymph-vascular space involvement
* S.D., standard deviation
* one factor ANOVA test, Mann-Whitney U test
NS, not significant

p=0.0041). In six tumors with positive lymph nodes, the heapranase expression was observed as being significantly high compared to tumors with negative lymph nodes (0.526±0.336 vs. 0.159±0.230, p=0.0049). In terms of depth of myometrial invasion, peritoneal cytology, ovarian metastasis, and cervical invasion, we observed no significant difference in the heparanase expression assessed using RT-PCR.

### *Microvessel density (MVD) and cilnicopathological factors*

The mean values of the microvessel score ranged from 8 to 30 (mean±SD, 17.8±6.2). FIGO stage IIIa (22.7±6.3), and IIIc (20.8±4.7) tumors were found to have a significantly higher microvessel score compared with stage I (Ia; 14.8±5.7, Ib; 15.1±4.3, Ic; 19.2±8.8) tumors. Tumors with a positive LVSI (22.5±5.9), grade 2/3 (21.7±4.8), lymph node metastasis (22.0±5.3), and ovarian metastasis (23.8±5.4) showed a significantly high microvessel score compared to those lacking these factors. The relationship between microvessel score and clinicopathological variables, and p values are shown in Table 2.

### *Correlation between heparanase mRNA expression and MVD*

We used Spearman's correlation coefficient by rank. Figure 2 demonstrates a strong correlation between heparanase expression and MVD (correlation coefficient $r^2$=0.475, Spearmann's provability p=0.0001).

### *IHC analysis was performed to analyze heparanase expression*

As shown in Figures 3 to 5, heparanase was abundantly and equally expressed in both the cytoplasm and the cell membrane of the cells in endometrial cancer (Figure 3, x40), and no heparanase expression was detected (Figure 4, x40). Strong heparanase -positive staining was also seen at the apex of cancer invasion into myometrium (Figure 5). Of specimens staining positive for heparanase, the majority expressed heparanase uniformly throughout the tumor. The IHC positive for heparanase in each clnicopathological factors are presented in Table 3. Tumors with deep myometrial invasion (depth c) expressed significantly higher heparanase positive rate compared with those in depth a or b tumors (p=0.0026). Grade 2 and 3 tumors showed a higher heparanase expression than those of grade 1 tumor, significantly (p=0.0135). In respects to LVSI, tumors with positive LVSI had a significantly higher expression rate of heparanase than those with negative LVSI (p=0.0028). In six tumors with positive lymph nodes, the heparanase expression was observed as being higher compared to tumors with negative lymph nodes, which

**Table 2 :** The correlation between microvessel count and clinicopathological factors

| Variables | No.of patients | Mean±S.D.[b] | P-value[c] |
|---|---|---|---|
| FIGO stage | | | |
| IA | 6 | 14.8±5.7 | |
| IB | 27 | 15.1±4.3 | p=0.005 |
| IC | 7 | 19.2±8.7 | |
| II | 1 | 16 | p=0.027 |
| IIIa | 4 | 22.7±6.3 | |
| IIIc | 7 | 20.8±4.7 | |
| Tumor grade | | | *p*=0.003 |
| 1 | 40 | 16.1±6.0 | |
| 2+3 | 12 | 21.7±4.8 | |
| Myometrial invasion | | | NS |
| a+b | 36 | 16.7±5.4 | |
| c | 16 | 19.8±7.1 | |
| LVSI[a] | | | *p*=0.001 |
| Yes | 18 | 22.5±5.9 | |
| No | 34 | 15.5±4.9 | |
| Lymph node metastasis | | | *p*=0.038 |
| Yes | 6 | 22.0±5.3 | |
| No | 46 | 16.9±6.0 | |
| Peritoneal cytology | | | NS |
| Yes | 2 | 21.8±6.4 | |
| No | 50 | 17.1±5.9 | |
| Ovarian metastasis | | | *p*=0.030 |
| Yes | 4 | 23.8±5.4 | |
| No | 48 | 17.1±5.9 | |
| Cervical invasion | | | NS[d] |
| Yes | 5 | 21.5±7.5 | |
| No | 47 | 17.3±5.9 | |

[a] LVSI, lymph-vasucular space involvement
[b] S.D.,standard deviation
[c] one factor ANOVA test, Mann-Whitney U test
[d] NS, not significant

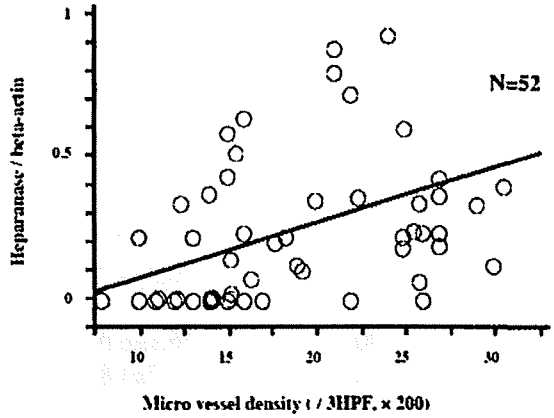


**Figure 2:** The correlation between ratios of heparanase/β-actin expression and microvesel score in endometial cancer as evaluated using the Spearmann's correlation test. Tumor MVD was correlated with the level of the ratios of heparanase/β-actin expression in endometrial cancer with correlation coefficient $r^2$=0.475, and Spearmann's provability; p=0.0001.

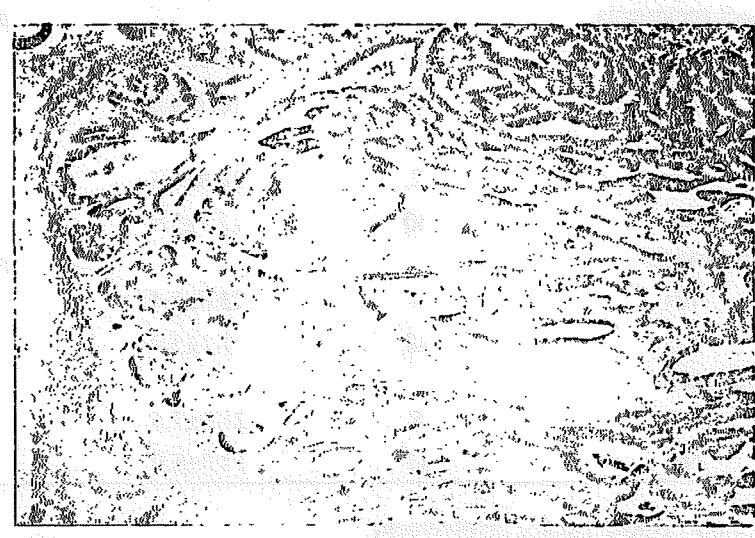

**Figure 3:** Heparanase was abundantly and equally expressed in both the cytoplasm and the cell membrane of the cells in endometrial cancer (x40). Of specimens staining positive for heparanase, the majority expressed heparanase uniformly throughout the tumor.

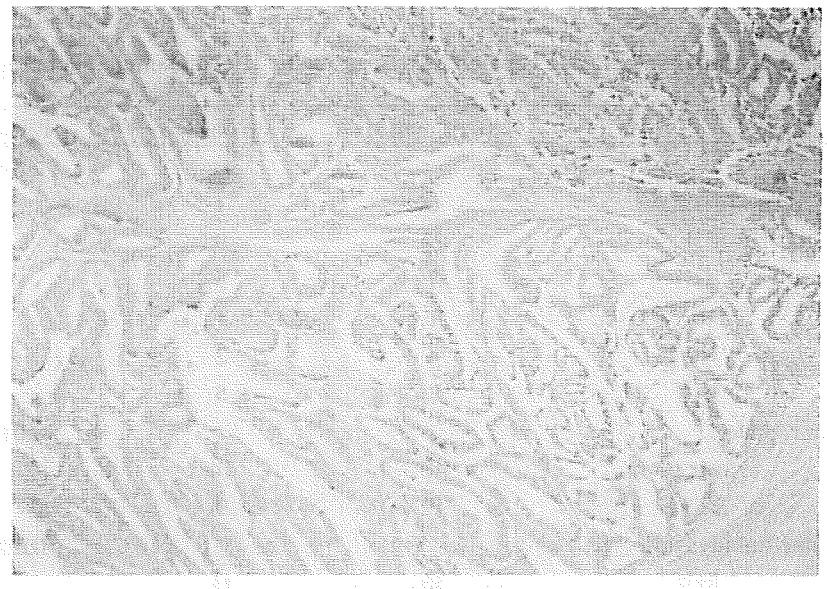

**Figure 4:** No heparanase expression was detected (x40)

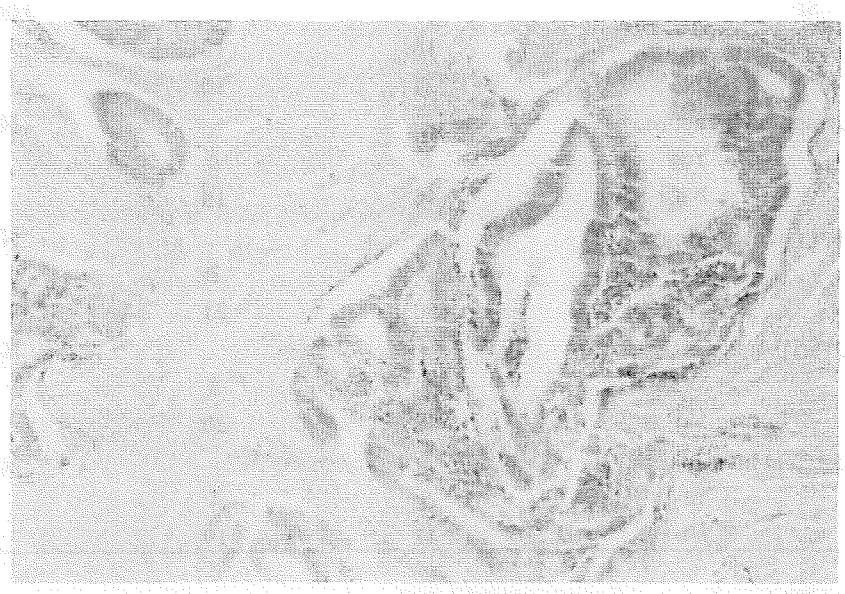

**Figure 5:** Strong heparanase -positive staining was also seen at the apex of cancer invasion into myometrium (x40).

Table 3: Correlation between heparanase expression by IHC and clinicopathological factors

| Variables | No. of patients | Hep positive | P-value[b] |
|---|---|---|---|
| FIGO stage | | | NS |
| IA | 6 | 1 | |
| IB | 27 | 9 | |
| IC | 7 | 5 | |
| II | 1 | 1 | |
| IIIa | 4 | 2 | |
| IIIc | 7 | 5 | |
| Tumor grade | | | 0.0135 |
| 1 | 40 | 14 | |
| 2+3 | 12 | 9 | |
| Myometrial invasion | | | 0.0026 |
| a+b | 36 | 11 | |
| c | 16 | 12 | |
| LVSI[a] | | | 0.0028 |
| Yes | 18 | 13 | |
| No | 34 | 10 | |
| Lymph node metastasis | | | NS |
| Yes | 6 | 4 | |
| No | 46 | 19 | |
| Peritoneal cytology | | | NS |
| Yes | 2 | 2 | |
| No | 50 | 21 | |
| Ovarian metastasis | | | NS |
| Yes | 4 | 3 | |
| No | 48 | 20 | |
| Cervical invasion | | | NS |
| Yes | 5 | 4 | |
| No | 47 | 19 | |

[a] LVSI, lymph-vasuenlar space involvement

[b] Fisher's exact test

NS, not significant

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.

## *Discussion*

Tumor 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 [30, 31]. Expression of heparanase, which degrades heparan sulfate correlates with the metastatic potential of tumor cells, and treatment with heparanase inhibitors markedly reduces the incidence of metastasis in experimental animals [1, 32, 33]. Heparanase may thus facilitate both tumor cell invasion and neovascularization, two critical steps in tumor progression. Our results showed that heparanase mRNA was detectable in 50% of the endometrial cancers [27, 28] according to the method of Vlodavsky et al. [1] and that its expression was closely associated with LVSI, lymph node metastasis, and tumor grade. The enzyme may play an important role in metastatic spread of the cancerous cells, as previously reported in several types of cancer, such as melanoma [3], gastric cancer [4], bladder cancer [5], pancreas cancer [6,7], hematogeneous [8], oral carcinoma [9], hepatocellular carcinoma [10], and colon cancer [11].

Heparin-binding angiogenic proteins are stored as a complex with heparan sulfate in the microenvironment of tumors. These proteins are released and can induce new capillary growth when heparan sulfate is degraded by heparanase. Heparanase may influence the bioavailability of different growth factors including FGFs, VEGF, HGF, and PDGF, which are stored in HS and possess HS-binding sequences [17, 18, 34, 35]. It is quite reasonable to assume that the release of such growth factors may influence tumor growth and angiogenesis. In hepatocellular carcinoma, the expression of heparanase enhances growth, invasion, and angiogenesis of the tumor, and bFGF seems to be a potent angiogenic factor [10]. In our study, we tested whether or not heparanase could influence tumor vascularity, and whether there was a direct correlation between heparanase expression and tumor angiogenesis in endometrial cancer [28]. El-Assal et al. [36] have previously demonstrated that different angiographic findings were not directly correlated with the MVD in hepatocellular carcinoma, because the degree of tumor stain is likely to be influenced by different factors, such as arterial supply, specificity of the canulation, lymphatic and venous drainage, and the amount of the contrast materials, besides the

MVD. Accordingly, we used MVD as a direct index for tumor neovascularization. Our study demonstrated that MVD was closely associated with LVSI, lymph node metastasis, ovarian metastasis, and tumor grade as well as heparanase expression. Furthermore, we found that the presence of positive correlation between heapranase expression and endometrial cancer angiogenesis in the present study. Tumors with strong heparanase expression had a significantly higher MVD compared with weak heparanase expression tumors. We can conclude that heparanase expression plays a pivotal role not only in the tumor growth and invasion but also in the angiogenesis of endometrial cancer [27,28].

The degree of histologic differentiation of endometrial cancer has long been accepted as one of the most sensitive indicators of prognosis. Also, the grade of tumor correlates with other prognostic factors. As the tumor loses its differentiation, the chances of survival decrease [37]. Interestingly in our study, as the tumor becomes less differentiated, both the heparanase expression and the MVD increase [27-29]. This may be one of the reasons that the histological tumor grade in endometrial cancer is a strong prognostic variable.

In conclusion, our study demonstrated the biological importance of heparanase expression in endometrial cancer. The expression of heparanase was found to influence different malignant behaviors in endometrial cancer, including lymph-vascular space involvement, lymph node metastasis and angiogenesis.

## References


1. Vlodavsky I, Friedmann Y, Elkin M, Aingorn H, Atzmon R, Ishai-Michaeli R, Bitan M, Pappo O, Peretz T, Michal I, Spector L, Pecker I., 1999. Nat Med 5, 793-802.
2. Hulett MD, Freeman C, Hamdorf BJ, Baker RT, Harris MJ, Parish CR., 1999. Nat Med 5, 803-809.
3. Marchetti D, Li J, Shen R., 2000. Cancer Res 60, 4767-4770.
4. Endo K, Maejara U, Baba H, Tokunaga E, Koga T, Ikeda Y, Toh Y, Kohnoe S, Okamura T, Nakajima M, Sugimachi K., 2001. Anticancer Res 21, 3365-3369.
5. Gohji K, Hirano H, Okamoto M, Kitazawa S, Toyoshima M, Dong J, Katsuoka Y, Nakajima M., 2001. Int J Cancer 95, 295-301.
6. Rohloff J, Zinke J, Schoppmeyer K, Tannapfel A, Witzigmann H, Mossner J, Wittekind C, Caca K., 2002. Br J Cancer 86, 1270-1275.
7. Koliopanos A, Friess H, Kleeff J, Shi X, Liao Q, Pecker I, Vlodavsky I,

Zimmermann A, Buchler MW., 2001. Cancer Res 61, 4655-4659.

8. Bitan M, Polliack A, Zecchina G, Nagler A, Friedmann Y, Nadav L, Deutsch V, Pecker I, Eldor A, Vlodavsky I, Katz BZ., 2002. Exp Hematol 30, 34-41.
9. Ikuta M, Podyma KA, Maruyama K, Enomoto S and Yanagishita M., 2001. Oral Oncol 37, 177-184.
10. El-Assal ON, Yamanoi A, Ono T, Kohno H, Nagasue N., 2001. Clin Cancer Res 7, 1299-1305.
11. Friedmann Y, Vlodavsky I, Aingorn H, Aviv A, Peretz T, Pecker I, Pappo O., 2000. Am J Pathol 157, 1167-1175.
12. Ruoslahti E, Yamaguchi Y., 1991. Cell 64, 867-869.
13. Rapraeger AC, Krufka A, Olwin BB., 1991. Science 252, 1705-1708.
14. Aviezer D, Hecht D, Safran M, Eisinger M, David G, Yayon A., 1994. Cell 79, 1005-1013.
15. Faham S, Hileman RE, Fromm JR, Linhardt RJ, Rees DC., 1996. Science 271, 1116-1120.
16. Park JE, Keller GA, Ferrara N., 1993. Mol Biol Cell 4, 1317-1326.
17. Folkman J, Klagsbrun M, Sasse J, Wadzinski M, Ingber D, Vlodavsky I., 1988. Am J Pathol 130, 393-400.
18. Raines EW, Ross R., 1992. J Cell Biol 116, 533-543.
19. Parish CR, Freeman C, Brown KJ, Francis DJ, Cowden WB., 1999. Cancer Res 59, 3433-3441.
20. Elkin M, Ilan N, Ishai-Michaeli R, Friedmann Y, Papo O, Pecker I, Vlodavsky I., 2001. FASEB J 15, 1661-1663.
21. Vlodavsky I, Friedmann Y., 2001. J Clin Invest 108, 341-347.

21. Wolfson AH, Sightler SE, Markoe AM, Schwade JG, Averette HE, Ganjei P, Hilsenbeck SG., 1992. Gynecol Oncol 45, 142-146.
23. Greven KM, Lanciano RE, Corn B, Case D, Randall ME., 1993. Cancer 71, 3697-3702.
24. Aoki Y, Kase H, Watanabe M, Sato T, Kurata H, Tanaka K., 2001. Gynecol Oncol 83, 1-5.
25. Watanabe M, Aoki Y, Kase H, Fujita K, Tanaka K., 2003. Int J Gynecol Cancer 13, 38-41.
26. Aoki Y, Watanabe M, Amikura T, Obata H, Sekine M, Yahata T, Fujita K, Tanaka K., 2004. Gynecol Oncol 94, 333-339.
27. Watanabe M, Aoki Y, Kase H, Tanaka K., 2002. Int J Gynecol Cancer 12, 632-633.

28. Watanabe M, Aoki Y, Kase H, Tanaka K., 2003. Gynecol Obstet Invest 56, 77-82.

29. Inamine M, Nagai Y, Hirakawa M, Mekaru K, Yagi C, Masamoto H, Aoki Y., 2008. J Obstet Gynaecol 28, 634-637.

30. Lyon M, Deakin JA, Mizuno K, Nakamura T, Gallagher JT., 1994. J Biol Chem 269, 11216-11223

31. Klein G, Conzelmann S, Beck S, Timpl R, Muller CA., 1995. Matrix Biol 14, 457-465.

32. Nakajima M, Irimura T, Di Ferrante N, Nicolson GL., 1984. J Biol Chem 259, 2283-2290.

33. Toyoshima M, Nakajima M., 1999. J Biol Chem 1274, 24153-24160.

34. Vlodavsky I, Korner G, Ishai-Michaeli R, Bashkin P, Bar-Shavit R, Fuks Z., 1990. Cancer Metastasis Rev 9, 203-226.

35. Mizuno K, Inoue H, Hagiya M, Shimizu S, Nose T, Shimohigashi Y, Nakamura T., 1994. J Biol Chem 269, 1131-1136.

36. El-Assal ON, Yamanoi A, Soda Y, Yamaguchi M, Igarashi M, Yamamoto A, Nabika T, Nagasue N., 1998. Hepatology 27, 1554-1562.

37. Creasman WT, Morrow CP, Bundy BN, Homesley HD, Graham JE, Heller PB., 1998. Cancer 60, 2035-2041.

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

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**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-$\mu$m) 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.

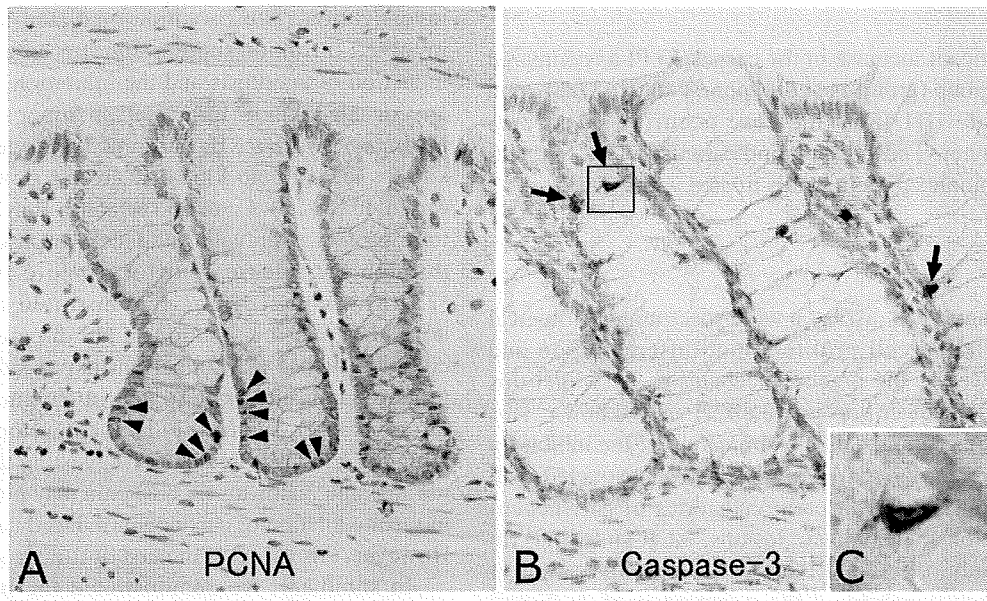


Figure 1. Representative results of immunohistochemical staining of PCNA (A) and caspase-3 (B and C) in the rat colonic epithelium. PCNA immunoreactivity is positive in the nucleus (arrow heads). Caspase-3-positive cells are mainly localized in the upper half of the crypt (arrows), and immunoreactivity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

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## References

1. Okazaki T, Bielawska A, Bell RM and Hannun YA: Role of ceramide as a lipid mediator of 1 alpha,25-dihydroxyvitamin D3-induced HL-60 cell differentiation. J Biol Chem 265: 15823-15831, 1990.

2. Riboni L, Prinetti A, Bassi R, Caminiti A and Tettamanti G: A mediator role of ceramide in the regulation of neuroblastoma Neuro2a cell differentiation. J Biol Chem 270: 26868-26875, 1995.
3. Obeid LM, Linardic CM, Karolak LA and Hannun YA: Programmed cell death induced by ceramide. Science 259: 1769-1771, 1993.
4. Cifone MG, De Maria R, Roncaioli P, *et al*: Apoptotic signaling through CD95 (Fas/Apo-1) activates an acidic sphingomyelinase. J Exp Med 180: 1547-1552, 1994.
5. Haimovitz-Friedman A, Kan CC, Ehleiter D, *et al*: Ionizing radiation acts on cellular membranes to generate ceramide and initiate apoptosis. J Exp Med 180: 525-535, 1994.
6. Bird RP: Observation and quantification of aberrant crypts in the murine colon treated with a colon carcinogen: preliminary findings. Cancer Lett 37: 147-151, 1987.
7. Bird RP: Role of aberrant crypt foci in understanding the pathogenesis of colon cancer. Cancer Lett 93: 55-71, 1995.
8. Corpet DE and Tache S: Most effective colon cancer chemopreventive agents in rats: a systematic review of aberrant crypt foci and tumor data, ranked by potency. Nutr Cancer 43: 1-21, 2002.
9. Caderni G, Femia AP, Giannini A, *et al*: Identification of mucin-depleted foci in the unsectioned colon of azoxymethane-treated rats: correlation with carcinogenesis. Cancer Res 63: 2388-2392, 2003.
10. Femia AP, Bendinelli B, Giannini A, *et al*: Mucin-depleted foci have beta-catenin gene mutations, altered expression of its protein, and are dose- and time-dependent in the colon of 1,2-dimethylhydrazine-treated rats. Int J Cancer 116: 9-15, 2005.
11. Yoshimi N, Morioka T, Kinjo T, *et al*: Histological and immunohistochemical observations of mucin-depleted foci (MDF) stained with Alcian blue, in rat colon carcinogenesis induced with 1,2-dimethylhydrazine dihydrochloride. Cancer Sci 95: 792-797, 2004.
12. Femia AP, Dolara P and Caderni G: Mucin-depleted foci (MDF) in the colon of rats treated with azoxymethane (AOM) are useful biomarkers for colon carcinogenesis. Carcinogenesis 25: 277-281, 2004.
13. Pierre Freeman A, Tache S, van der Meer R and Corpet DE: Beef meat and blood sausage promote the formation of azoxymethane-induced mucin-depleted foci and aberrant crypt foci in rat colons. J Nutr 134: 2711-2716, 2004.
14. Dillehay DL, Webb SK, Schmelz EM and Merrill AH Jr: Dietary sphingomyelin inhibits 1,2-dimethylhydrazine-induced colon cancer in CF1 mice. J Nutr 124: 615-620, 1994.
15. Schmelz EM, Bushnev AS, Dillehay DL, Liotta DC and Merrill AH Jr: Suppression of aberrant colonic crypt foci by synthetic sphingomyelins with saturated or unsaturated sphingoid base backbones. Nutr Cancer 28: 81-85, 1997.
16. Schmelz EM, Dillehay, DL, Webb SK, Reiter A, Adams J and Merrill AH Jr: Sphingomyelin consumption suppresses aberrant colonic crypt foci and increases the proportion of adenomas versus adenocarcinomas in CF1 mice treated with 1,2-dimethylhydrazine: implications for dietary sphingolipids and colon carcinogenesis. Cancer Res 56: 4936-4941, 1996.
17. Schmelz EM, Sullards MC, Dillehay DL and Merrill AH Jr: Colonic cell proliferation and aberrant crypt foci formation are inhibited by dairy glycosphingolipids in 1,2-dimethylhydrazine-treated CF1 mice. J Nutr 130: 522-527, 2000.
18. Symolon H, Schmelz EM, Dillehay DL and Merrill AH Jr: Dietary soy sphingolipids suppress tumorigenesis and gene expression in 1,2-dimethylhydrazine-treated CF1 mice and ApcMin/+ mice. J Nutr 134: 1157-1161, 2004.
19. Inamine M, Suzui M, Morioka T, *et al*: Inhibitory effect of dietary monoglucosyl-ceramide 1-O-beta-glucosyl-N-2'-hydroxy-arachidoyl-4,8-sphingadienine on two different categories of colon preneoplastic lesions induced by 1,2-dimethylhydrazine in F344 rats. Cancer Sci 96: 876-881, 2005.
20. Hall PA and Woods AL: Immunohistochemical markers of cellular proliferation: achievements, problems and prospects. Cell Tissue Kinet 23: 505-522, 1990.
21. Gown AM and Willingham MC: Improved detection of apoptotic cells in archival paraffin sections: immunohistochemistry using antibodies to cleaved caspase 3. J Histochem Cytochem 50: 449-454, 2002.
22. Schmelz EM, Bushnev AS, Dillehay DL, Sullards MC, Liotta DC and Merrill AH Jr: Ceramide-beta-D-glucuronide: synthesis, digestion, and suppression of early markers of colon carcinogenesis. Cancer Res 59: 5768-5772, 1999.
23. Ahn EH and Schroeder JJ: Sphingoid bases and ceramide induce apoptosis in HT-29 and HCT-116 human colon cancer cells. Exp Biol Med 227: 345-353, 2002.
24. Ogretmen B and Hannun YA: Biologically active sphingolipids in cancer pathogenesis and treatment. Nat Rev Cancer 4: 604-616, 2004.
25. Yang J, Yu Y, Sun S and Duerksen-Hughes PJ: Ceramide and other sphingolipids in cellular responses. Cell Biochem Biophys 40: 323-350, 2004.
26. Kolesnick R: The therapeutic potential of modulating the ceramide/sphingomyelin pathway. J Clin Invest 110: 3-8, 2002.
27. Heinrich M, Wickel M, Schneider-Brachert W, *et al*: Cathepsin D targeted by acid sphingomyelinase-derived ceramide. EMBO J 18: 5252-5263, 1999.
28. Selzner M, Bielawska A, Morse MA, *et al*: Induction of apoptotic cell death and prevention of tumor growth by ceramide analogues in metastatic human colon cancer. Cancer Res 61: 1233-1240, 2001.
29. Schmelz EM, Roberts PC, Kustin EM, *et al*: Modulation of intracellular beta-catenin localization and intestinal tumorigenesis in vivo and in vitro by sphingolipids. Cancer Res 61: 6723-6729, 2001.

# Endometrial Cancer State of the Science Meeting

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**Abstract:** There is a pressing need to improve our understanding of endometrial cancer (EC) and uterine carcinosarcoma and to develop new treatment strategies to improve outcomes. In recognition of this, a State of the Science meeting on EC was held last November 28 and 29, 2006, in Manchester, United Kingdom. The meeting was cosponsored by the National Cancer Research Institute (UK), the National Cancer Institute (US), and the Gynecological Cancer Intergroup.

The objectives of the meeting were as follows:

1. To review current knowledge and understanding of EC and its treatments.
2. To identify key issues for translational research and clinical trials.
3. To identify the most important trials for women with endometrial carcinoma and uterine carcinosarcoma, both those already underway or to be done, for which the Gynecological Cancer Intergroup might facilitate international cooperation.

**Key Words:** Endometrial cancer, Clinical trials, Translational research

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Endometrial cancer (EC), the second most common gynecologic cancer worldwide, has now become the most common gynecologic cancer in developed countries. Its rising incidence is related to increasing life expectancy, tamoxifen use, and the epidemic of obesity. The last is also responsible for comorbidity, notably adult-onset diabetes and hypertension. Together, comorbidity and obesity present challenges in delivering optimal therapy for many women with EC. The rising incidence of EC has been associated with a rising death rate. Although the prognosis of early disease is good with a survival rate of 80%, those with very high-risk disease and advanced disease at presentation have a survival rate below 50% with very little gain in therapeutic efficacy during the past 30 years. This lack of progress in treatment is, in part, related to our limited understanding of the molecular pathology of EC. There is a pressing need to improve our understanding of EC and to develop new treatment strategies to improve outcomes. In addition, compared with ovarian and cervical cancer, EC and uterine carcinosarcoma (CS) have been studied much less extensively. Fewer trials have been opened for women with these cancers, and accrual to those trials has been slow.

In recognition of this, a State of the Science meeting on EC was held last November 28 and 29, 2006 in Manchester, United Kingdom. The meeting was cosponsored by the National Cancer Research Institute (NCRI, UK), the National Cancer Institute (US), and the Gynecological Cancer Intergroup (GCIG). A multidisciplinary group of 75, drawing on surgeons, gynecologic oncologists, radiation (clinical) oncologists, medical oncologists, pathologists, translational scientists, and patient advocates from 18 countries and representing 14 trial groups attended.

The objectives of the meeting were as follows:

1. To review current knowledge and understanding of EC and its treatments.
2. To identify key issues for translational research and clinical trials.
3. To identify the most important trials for women with endometrial carcinoma and uterine CS, both those already underway or to be done, for which the GCIG might facilitate international cooperation.

The first half of the proceedings was dedicated to a series of presentations, which outlined our current knowledge. The second half of the meeting began with parallel sessions of early disease and advanced/recurrent disease to define staging, treatment, and translational research issues to lead to candidate clinical trials questions. This was followed by plenary discussion of the questions to be addressed in these candidate trials and an attempt to develop an international consensus of the most favored concepts for future development and international collaboration.

This paper reports the content and conclusions arising from this meeting.

## CURRENT KNOWLEDGE

### Molecular Pathology of EC

#### Endometrial Hyperplasia

There is broad agreement that type 1 (estrogen-related) EC progresses via a precursor lesion, atypical hyperplasia or endometrial intraepithelial neoplasia.[1] This has been clearly demonstrated

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