Abstract. Endocrine gland-derived vascular endothelial growth factor (EG-VEGF) has recently been identified as one of the vascular endothelial growth factors, and it is considered that the overexpression of EG-VEGF in colon cancer is related to hepatic metastasis. In this study, we report our recent novel findings of the involvement of EG-VEGF in cell invasion of colon cancer cells. Colon cancer cell lines (DLD-1 and HCT116) with high expression of prokineticin receptor (PK-R) 1 and 2 were stimulated with the EG-VEGF protein. Furthermore, Matrigel cell invasion assay was performed to examine the changes in cancer cell invasion. In addition, we investigated the mRNA expression of matrix metalloproteinase (MMP)-2, -7 and -9 in cancer cells. Finally, the EG-VEGF receptor on the colon cancer cell membrane was blocked by anti-PK-R1 and -PK-R2 antibodies to study whether cell invasion ability would be altered. In colon cancer cell lines where the expression of PK-R1 and 2 was confirmed, stimulation with EG-VEGF increased cell invasion a maximum of ~3-5 times. Furthermore, an increase in the mRNA and protein expression of MMP-2, -7 and -9 was observed. We also observed that the cell invasion rate decreased only after exposure to the anti-PK-R2 antibody. The study showed that the EG-VEGF protein may act on MMP-2, -7 and -9 via PK-R2 to strengthen cell invasion ability in colon cancer cell lines.

Introduction

At present, a major issue related to treating gastrointestinal cancers is dealing with metastasis, as this may determine patient prognosis. Among the different types of cancer, colon cancer is characterized by susceptibility to hematogenous metastasis, particularly hepatic metastasis. Successful management of hematogenous metastasis is considered vital for an improvement in the survival rate of these patients (1,2).

Colon cancer metastasis occurs possibly through the following mechanism: after detaching from the primary lesion, the cancer cells invade capillary vessels, pass through the portal system and greater circulatory system, spread across the whole body, adhere to vascular endothelial cells of a target organ, transmigrate and invade outside the blood vessels and grow in the metastatic lesion. Cancer cells have been clinically shown to undergo such invasive processes, and those cancer cells that have high invasive ability are considered likely to cause metastasis and poor prognosis (3). The endocrine gland-derived vascular endothelial growth factor (EG-VEGF) gene that was investigated in this study was identified by LeCouter et al in 2001 as an angiogenic growth factor for organs of the endocrine system. EG-VEGF consists of 305 amino acids with a molecular weight of 8.6 kDa. A mature EG-VEGF protein has 86 amino acids containing 10 cysteines, with 80% homology to a non-toxic protein purified from the venom of the black mamba snake, whereas homology to the vascular endothelial growth factor (VEGF) is weak (4). Recently, prokineticin receptor (PK-R) 1 and 2 were identified as EG-VEGF receptors and these receptors were shown to mediate physiological changes (5-7).

This study demonstrates our findings of a new mechanism by which signal transmission to matrix metalloproteinase (MMP) via PK-R2 occurs after stimulation with the EG-VEGF protein, thereby accelerating cell invasion in colon cancer cells.

Materials and methods

Cell culture. The human colon cancer cell lines, DLD-1 and HCT116, were cultured at 37°C in 5% CO₂ in RPMI-1640 medium containing 10% fetal bovine serum (8).

Antibody. The following antibodies were used: anti-human PK-R1 and PK-R2 Ab (Novus Biochemicals, Littleton, CO, USA).
**Immunohistostaining.** The cells were plated in 96-well plates at 1x10⁴ and incubated for 12 h. The cells were analyzed for protein expression using the streptavidin-biotin peroxidase method (9,10).

**Chemicals.** EG-VEGF proteins were dissolved in distilled water according to the manufacturer's instructions (Shenandoah Biotechnology, Inc., Warwick, PA, USA).

**Antibody treatment.** Cancer cells were plated in 10-cm dishes at 5x10⁵ and incubated for 12 h. The cells were treated with antibody at 10 µg/ml for 3 h.

**Tumor cell invasion assay.** Transwells (Biocoat Matrigel 6-well invasion chamber) with filters coated with an extracellular matrix (Matrigel) on the upper surface were purchased from BD Biosciences (San Jose, CA, USA). A complete medium was added to the bottom chamber to induce the invasion of the cells through the Matrigel. A serum-free medium with or without EG-VEGF protein was added to the cells (2x10⁵), and seeded to the top chamber. The Matrigel invasion chamber was incubated for 48 h at 37˚C with 5% CO₂. Non-invading cells were removed from the top of the Matrigel with a cotton-tipped swab. The number of invasive cells was determined by counting the stained cells. Cell numbers were counted with a hemocytometer (11).

**RNA extraction and RT-PCR analysis.** Total RNA was extracted from cells using Isogen (Wako, Osaka, Japan). The single-strand cDNA prepared from 3 µg of total RNA using PrimeScript RT reagent kit (Takara, Japan) was used as the template for the polymerase chain reaction (PCR) (12). The primers for PCR to amplify MMP-2 gene-coding regions were as follows: 5' primer MMP-2-AX, 5'-ACCCATTTACACCTACACCAAG-3'; 3' primer MMP-2-BX, 5'-GTATACCGCATCAAATTTTCCG. The primers for PCR to amplify MMP-7 gene-coding regions were as follows: 5' primer MMP-7-AX, 5'-TCTTTGGCCTACCTATAACTGG-3'; 3' primer MMP-7-BX, 5'-CTAGACTGTGCTACCATCCCTGTA-3'. The primers for PCR to amplify MMP-9 gene-coding regions were as follows: 5' primer MMP-9-AX, 5'-TGGGTACCGTACCATGACT-3'; 3' primer MMP-9-BX, 5'-GCCAGCCCACCCTCCACTCTTC-3'. GAPDH amplification was used as an internal PCR control with 5'-GGGGAGCAGATCTTTGACATT-3' as the sense primer and 5'-GACGTCGCGTACCTTGCGG-3' as the antisense primer. Thirty cycles of denaturation (94˚C, 1 min), annealing (50˚C, 1.5 min) and extension (72˚C, 2 min) were carried out in a thermal cycler (PTC-100, Programmable Thermal Controller; MJ Research Inc., MA, USA). PCR products (10 µl) were resolved by electrophoresis in 1.2% agarose gel. The sequencing was performed on PCR products showing the bands in RT-PCR analysis. Ethidium bromide staining of the gels identified a band of the MMP-2, -7 and -9 mRNA. To ensure reproducibility, all PCR amplifications were performed in triplicate (8,12).

**Statistical considerations.** Characteristics of the two treatment arms were compared using the Chi-square test. Values of P<0.05 were considered to indicate statistically significant results.

**Results**

**Expression levels of PK-R1 and PK-R2 in colon cancer cells.** Fig. 1 shows the stained image of colon cancer cell lines: DLD-1 and HCT116. Expression of PK-R1 and PK-R2 was expressed in both cancer cell lines.

**Invasive ability of the colon cancer cell lines.** Figs. 2 and 3 demonstrate the cell invasion after stimulation of colon cancer cells with the EG-VEGF protein. While the number of invasive HCT116 colon cancer cells was 117 on average, the number of invasive HCT116 cells after stimulation with EG-VEGF was 346 on average. Similarly, while the mean number of invasive DLD-1 colon cancer cells was 49, the mean number of invasive DLD-1 cells following stimulation with EG-VEGF was significantly higher at 288.
Expression of MMP-2, -7 and -9 mRNAs in colon cancer cell lines. Results of the expression of MMP-2, -7 and -9 mRNAs in the colon cancer cell lines stimulated with the EG-VEGF protein are shown in Fig. 4. Compared with the cell lines that were not stimulated with EG-VEGF protein, stimulation with EG-VEGF protein increased the expression of MMP-2, -7, and -9 mRNAs in both the HCT116 and DLD-1 cell lines, although the amount of increase was not the same for both cell lines.

Suppression of the invasive ability of colon cancer cell lines by anti-PK-R1 antibody. While 346 HCT116 colon cancer cells were invasive after EG-VEGF stimulation, 316 HCT116 cells were invasive following exposure to the anti-PK-R1 receptor antibody following EG-VEGF stimulation. While 331 DLD-1 cells were invasive following EG-VEGF stimulation, 275 DLD-1 cells were invasive after exposure to the anti-PK-R1 receptor antibody following EG-VEGF stimulation (Fig. 5). Thus, cell invasion was not significantly suppressed.

Suppression of the invasive ability of colon cancer cell lines by anti-PK-R2 antibody. While 346 HCT116 colon cancer cells were invasive after EG-VEGF stimulation, 119 HCT116 cells were invasive following exposure to anti-PK-R2 antibody followed by EG-VEGF stimulation. While 331 DLD-1 cells were invasive following EG-VEGF stimulation, 93 DLD-1 cells were invasive after exposure to the anti-PK-R2 receptor antibody followed by EG-VEGF stimulation (Fig. 5). Thus, cell invasion was significantly suppressed.

Discussion

The most serious life-threatening condition associated with malignant tumors is metastasis. Possible countermeasures include i) preventing cancer cells from leaving the primary lesion and reaching a target organ; and ii) suppressing the growth of lesions in the metastatic organs. An important phenomenon in the course of metastasis is angiogenesis. Angiogenesis intricately involves the platelet-derived growth factor (PDGF), VEGF and other growth factors as well as their binding status to receptors on an endothelial surface (13-16). Many reports have been published regarding the relationships between these factors and the development of malignant tumors. With the recent advancement of molecular biological techniques, the mechanism of angiogenesis has been elucidated, and various types of angiogenesis inhibitors have shown efficacy in clinical applications (17-22).

EG-VEGF investigated in this study is weakly homologous to and different from VEGF, a known angiogenic factor. According to previous studies, the expression of EG-VEGF in normal human tissue is limited to hormone-producing cells including ovary, testis and placenta, and does not occur in normal gastrointestinal membranes such as the stomach and colon (4). In connection with our investigations on primary lesions in colon cancer, we report the following. The prognosis of patients with positive expression of EG-VEGF mRNA is significantly worse than for patients with negative expression and intensification of EG-VEGF expression is related to angiogenesis and hepatic metastasis (23,24). EG-VEGF expression is also considered highly significant in other malignant tumors and is associated with metastasis of prostate cells.
cancer/neuroblastoma and the intensification of malignancy in pancreatic duct cancer (25-28). Furthermore, our study showed that EG-VEGF is related to cell invasion ability, which is an indicator of cell malignancy and an important element for the development of hematogenous, lymph node and peritoneal metastasis. The importance of EG-VEGF in malignant cells was thus indicated.

Although not many studies have been conducted on EG-VEGF in colon cancer, regarding the transmission system of EG-VEGF, it is known that information enters the cell via PK-R1 and 2 on the cell membrane, eventually inducing physiological phenomena and other various important events (5-7). To the best of our knowledge, our study showed for the first time that invasive ability of colon cancer cells increases with the stimulation of the EG-VEGF protein and that information of the EG-VEGF protein is possibly transmitted via the PK-R2, which we proved by inhibition of invasion by the anti-PK-R2 antibody. Furthermore, we examined the information transmission system in detail by focusing on the MMP family, which plays an important role in the degradation of the extracellular matrix at the time of migration from the primary lesion to the interstitium and to the inside of vascular channels (29,30). Among the MMP family, MMP-2, -7, and -9, are involved in the invasive ability of colon cancer cells (31,32). We examined MMP family members and found that stimulation with the EG-VEGF protein augmented the expression of MMP-2, -7 and -9 genes, suggesting the importance of the MMP family genes.

We showed that the EG-VEGF protein acts on the MMP family genes via the PK-R2 receptor on the cellular membrane, ultimately intensifying the cell invasion ability in colon cancer (Fig. 6).
References