Antisense Phosphorothioate Oligodeoxynucleic Acid for CD10 Suppresses Liver Metastasis of Colorectal Cancer

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Introduction

CD10 expression is associated with colorectal cancer (CRC) metastases, especially liver metastasis [1–4]. In gastric cancer and pancreatic endocrine cancer, CD10 expression is also associated with liver metastasis [5–7]. CD10 expression is observed in cancer stromal cells, which is associated with CRC invasion and metastasis [8]. Thus, CD10 is a relevant marker for liver metastasis of CRCs; however, the CD10 functions in CRCs are still unknown. We prompted to reveal the biological roles of CD10 for the liver metastasis and the therapeutic significance of CD10 targeting.

CD10 is a 90- to 110-kDa cell zinc-dependent membrane metalloendopeptidase also designated as neutral endopeptidase 24.11 (EC 3.4.24.11) [9]. CD10 is known as common acute lymphoblastic leukemia antigen (CALLA), which is widely used to define subgroups within B cell type acute lymphocytic leukemia [9, 10]. CD10 is expressed in various tissues including hematopoietic cells, epithelial cells and placenta [11–13]. Especially, in the intestinal mucosa, CD10 is located at the brush border of the luminal surface of the intestine. Furthermore, CD10 expression is recognized as a mucin phenotype of the small intestine in gastrointestinal mucosa [14]. In some reports, biological characterization of CD10 is examined in cancers. CD10 expression is associated with mutation of adenomatous polyposis coli gene in gastric cancer [15].
pancreatic endocrine cancer, CD10 is correlated with proliferative index, large tumor size and histological dedifferentiation [7]. CD10 expression in CRCs, malignant melanoma and oral cavity squamous cell carcinoma is associated with cancer invasion and progression [8, 16, 17].

In the present study, we revealed that CD10 plays biological roles in forming liver metastasis of CRC and that suppression of CD10 is effective to inhibit metastasis.

Materials and Methods

Cell Culture

HT29 human colon cancer cell line was purchased from Dai-nihon Pharmaceutical Co. (Tokyo, Japan). All cell lines were maintained in Dulbecco’s modified essential medium (Sigma Chemical Co., St. Louis, Mo., USA) containing 10% fetal bovine serum (Sigma Chemical Co.) under conditions of 5% CO2 in air at 37°C. (DL-3-mercapto-2-benzylpropanoyl)-glycine (Thiorphan) was purchased from Sigma Chemical Co.

Antisense Phosphorothioate (S)-Oligodeoxynucleotide Assay

A 20-mer S-oligodeoxynucleotide (ODN) composed of the antisense sequence of nucleotides 5–15 of human CD10 cDNA (referred to GenBank BC101658) was synthesized and purified by reverse-phase high-performance liquid chromatography (Sigma Genosys, Ishikari, Japan). The sequence of the CD10 antisense ODN was 5'-TTC TGA CTT GCC CAT AAC CT-3'. Random 18-mers were used as negative controls. Cells were pretreated with 3 µM antisense or random S-ODN for 6 days, with medium exchange and addition of antisense or random S-ODN every 2 days. The cells were then used in experiments. For using liposome, CD10 antisense S-ODN solution (6 µM, 2 ml) was added to empty liposome preparation (EL-C-01, DPPC: 26 mM; Nippon Oil & Fats Co., Tokyo, Japan).

Cell Growth

HT29 cells were seeded at a density of 10,000 cells per well in 12-well tissue culture plates. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Chemical Co.) was added to the culture medium at a concentration of 25 µg/ml 1 h before harvest. Harvested cell pellets were lysed with 1 ml dimethyl sulfoxide, and 200 µl of the lysate were examined at 620 nm. The experiments were performed in triplicate.

Cell Migration Assay

To evaluate cell motility, a wound healing assay was performed. HT29 cells were exposed to 3 µM antisense S-ODN for CD10 or random S-ODN for 6 days. Then they were re-seeded onto 3.5-cm culture dishes and continuously treated with antisense S-ODN for CD10 or random S-ODN. After 2 days, cells grown to subconfluence were scraped to make a cell-free area with sharp edges. Cells migrating into the scraped area were counted every 12 h after scraping. The experiments were performed in triplicate.

In vitro Invasion Assay

A modified Boyden chamber assay was performed to examine the in vitro invasion of colon cancer cells, HT29 cells treated with antisense S-ODN for CD10 or random S-ODN. Polycarbonate filters (pore size 3 µm, diameter 5 mm) were glued to collagen-type IV inserts (Becton-Dickinson Labware, Bedford, Mass., USA), which were placed in the wells of 24-well tissue culture plates. Cells were suspended in 500 µl of regular medium and placed in the upper part of the chamber. The lower part of the chamber was filled with regular medium. After 24-hour incubation at 37°C, the filters were carefully removed from the inserts, stained with hematoxylin for 10 min and mounted on microscopic slides. The number of stained cells was counted in whole inserts at 100× magnification. Invasion activity was quantified by the average of cells per insert well. The experiments were performed in triplicate.

Animal Model

BALB/c nu-nu athymic mice were purchased from Japan SLC Inc. (Shizuoka, Japan). The mice were maintained according to the institutional guidelines approved by the Committee for Animal Experimentation of Nara Medical University, in accordance with the current regulations and standards of the Ministry of Health, Labour and Welfare. The mice were used according to the institutional guidelines when they were 5 weeks old. Cells were briefly trypsinized and washed with Hanks balanced saline solution (HBSS), 3 times. The cells suspended by HBSS were injected into the spleen by 1 × 106 in 50 µl HBSS in each mouse. Ten mice were injected for each group. Then, the mice were sacrificed to count the number and size of metastatic foci in the liver. For measuring the number and size of the metastatic foci in the liver, the liver was dissected at 4 weeks after inoculation of cancer cells. Metastatic tumors were observed macroscopically before fixation, and the number of the foci and each size of the focus were measured.

Fig. 1. Effects of CD10 antisense S-ODN on growth, migration, invasion and apoptosis in HT29 human colon carcinoma cells. a, b CD10 protein levels by CD10 antisense S-ODN detected by immunoblotting (a) and immunocytochemistry (b). c Cell growth. d–f Cell migration examined by wound healing assay. g–i In vitro invasion assay using type IV collagen-coated membrane. Arrowhead indicates cells invading into the membrane; arrow indicates cells on the membrane. j–l Colony formation assay of survived HT29 cells from nitric oxide-induced apoptosis by contrasted microscopy examination. Error bars show SD from 3 independent assays. R = Random S-ODN; AS = CD10 antisense S-ODN.
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Treatment (h)

Cell count (×10,000)

0 2 4 6 8 10 12 14 16 18 20

Migration (cells per mm)

0 10 20 30 40 50 60 70 80

Invasion (cells per well)

0 10 20 30 40 50 60

Colony number (per well)

0 10 20 30 40 50 60

R

AS

R

AS

p = 0.0078

p = 0.0286

p = 0.0342

p = 0.0286

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Immunoblot Analysis

Whole-cell lysates were prepared as described previously [18]. Fifty-microgram lysates were subjected to immunoblot analysis in 12.5% sodium dodecyl sulfate polyacrylamide gels followed by electrotransfer to nitrocellulose filters. The filters were incubated with primary antibodies, and then with peroxidase-conjugated IgG antibody (Medical and Biological Laboratories, Nagoya, Japan). Primary antibodies used were anti-CD10 antibody (Novocastra Laboratories, Newcastle upon Tyne, UK), anti-ERK1/2 (Chemicon International, Temecula, Calif., USA), anti-phosphorylated ERK1/2 (Santa Cruz Biotechnology Inc., Santa Cruz, Calif., USA), anti-JNK (JNK1, p46; Santa Cruz Biotechnology Inc.), anti-phosphorylated JNK (Santa Cruz Biotechnology Inc.), anti-p38 (clone A-12; Santa Cruz Biotechnology Inc.), anti-phosphorylated p38 (clone D-8; Santa Cruz Biotechnology Inc.), anti-EGFR (clone 1005; Santa Cruz Biotechnology Inc.) and anti-phosphorylated EGFR (phospho-Tyr992; Cell Signaling Technology, Beverly, Mass., USA). A γ-tubulin antibody was used to assess the levels of protein loaded per lane (Oncogene Research Products, Cambridge, Mass., USA). The immune complex was visualized with an ECL Western blot detection system (Amersham, Aylesbury, UK).

Immunohistochemistry

Consecutive 4-μm sections were immunohistochemically stained using the immunoperoxidase technique described previously [19]. Anti-CD10 antibody (Novocastra Laboratories), and anti-cytokeratin 20 antibodies (Progen Biotechnik, Heidelberg, Germany) were used at a concentration of 0.5 μg/ml. Secondary antibodies (Medical and Biological Laboratories, Nagoya, Japan) were used at a concentration of 0.2 μg/ml. Specimens were color developed with diaminobenzidine hydrochloride (Dako Corp., Carpinteria, Calif., USA). Meyer’s hematoxylin (Sigma Chemical Co.) was used for counterstaining.
Statistical Analysis
The two-tailed χ² test and Mann-Whitney U test were used. Statistical significance was defined as a two-sided p < 0.05.

Results

Effect of CD10 Repression on Growth and Invasion in HT29 Cells

HT29 human colon cancer cells expressed CD10, which was repressed by CD10 antisense S-ODN treatment (fig. 1a, b). CD10 antisense S-ODN treatment reduced cell growth, in vitro invasion and colony formation, but not migration (fig. 1c–l). HT29 cells with CD10 antisense S-ODN treatment showed decreased cell growth to 58% of that in HT29 cells with random S-ODN treatment (p = 0.0078). CD10 antisense S-ODN treatment decreased invasion into type IV collagen membrane to 27% of that in HT29 cells with random S-ODN treatment (p = 0.0286). In HT29 cells with CD10 antisense S-ODN treatment, colony formation after nitric oxide-induced apoptosis was decreased to 24% of that in HT29 cells with random S-ODN treatment (p = 0.0342).

Effect of CD10 Repression on Liver Metastasis of HT29 Cells

Repression of CD10 gene by CD10 antisense S-ODN treatment inhibited liver metastasis in the nude mice model (fig. 2). HT29 cells treated with CD10 antisense S-ODN for 6 days decreased cell embedding in the liver sinusoids after the intrasplenic inoculation to 25% of that in HT29 cells treated with random S-ODN (p = 0.0003). At 4 weeks after inoculation, established liver metastasis was examined (fig. 2c–f). HT29 cells with CD10 antisense S-ODN treatment decreased the number and size of metastatic foci in the liver to 66 and 48%, respectively, of those in HT29 cells treated with random S-ODN (p = 0.0004 and p = 0.0022, respectively).

Effect of Met-Enkephalin on Mitogen-Activated Protein Kinase Signals in HT29 Cells

To elucidate the effect of CD10 antisense S-ODN on HT29 cells, phosphorylation status of mitogen-activated protein kinase (MAPK) was examined (fig. 3). Treatment with CD10 antisense S-ODN did not affect the protein levels of ERK1/2, JNK, p38 and EGFR. Treatment with CD10 antisense S-ODN decreased the phosphorylated form of ERK1/2 to 68% of that in random S-ODN-treated cells, whereas the phosphorylated form of p38 was increased to 312% of that in random S-ODN-treated cells. We also examined the expression of EGFR, which is one of the major activators of MAPK. EGFR protein levels were not affected by treatment with CD10 antisense S-ODN, whereas the phosphorylated form of EGFR was decreased to 55% of that in random S-ODN-treated cells.

Effect of Liposome-Capsulated CD10 Antisense S-ODN on Liver Metastasis of HT29 Cells

Finally, we examined effects of liposome-capsulated CD10 antisense S-ODN on prevention of liver metastasis and inhibition of metastatic foci growing (fig. 4). Intraperitoneal administration of liposome-capsulated CD10 antisense S-ODN from cancer cell inoculation into the spleen suppressed formation of liver metastasis (fig. 4a). In the next step, liposome-capsulated CD10 antisense S-ODN was administrated from 4 weeks after inoculation of HT29 cells into the spleen. At the beginning of the administration, metastatic foci grew to 4.3 ± 0.8 mm in diameter. Growth of the metastatic foci was inhibited by liposome-capsulated CD10 antisense S-ODN (fig. 4b).
CD10 is associated with CRC progression and metastases [1–3, 8]; however, the role of CD10 expression had not been defined as a surrogate marker or a biological activator of disease progression. In the present study, we confirmed that the CD10 expression was substantially associated with liver metastasis using CD10 antisense S-ODN in in vitro and a mouse liver metastasis model. In these experiments, CD10 repression inhibited cell growth, invasion and survival in CD10-positive HT29 human colon cancer cells, and decreased embedding and metastasis of HT29 cells in the mice liver.

MAPK activation is well known to mediate cell growth, invasion, and survival. We then examined the effect of CD10 repression on MAPK phosphorylation. CD10 antisense S-ODN treatment increased ERK1/2 phosphorylation, which might be associated with cell growth and invasion [20]. In contrast, CD10 antisense S-ODN treatment decreased p38 phosphorylation. We confirmed that p38 inhibition decreased apoptosis in HT29 cells (data not shown). EGFR is a major activator of MAPK in CRCs [21]. We then examined the effect of CD10 antisense S-ODN treatment on EGFR activation. CD10 antisense S-ODN treatment decreased phosphorylation levels of EGFR. The finding suggests that CD10 might be associated with EGFR activation.

Substrates of CD10 involve wide range of neuropeptides such as substance P, bradykinin, bombesin, Leu-enkephalin and Met-enkephalin [9, 22, 23]. Among these neuropeptides, substance P and enkephalin involve MAPK signals [24, 25]. Substance P transactivates EGFR [26], whereas enkephalin-induced EGFR transactivation depends on integrin signals [27]. The G6 glioma cell line, which expresses enkephalin receptor (δ-opioid receptor) and EGFR, shows inhibition of EGFR activation by activated δ-opioid receptor, whereas EGFR does not inhibit δ-opioid receptor [28]. Thus, enkephalin degradation by CD10 might provide a pro-tumoral effect through activation of EGFR. The tumor growth is a balance of cell proliferation and apoptosis. CD10 might affect the signal diversity in MAPK activation by EGFR to increase growth and decrease apoptosis. Subsequently, CD10 enhances tumor growth and metastasis.

In the present study, we used liposome-capsulated S-ODN. Liposome enhances delivery and transfer of the liposomal contents into target cells [29]. Especially, liposome tends to accumulate in the liver [30]. Thus, liposome is a suitable vehicle to deliver CD10 antisense S-ODN for treatment of liver metastasis. Anti-tumoral effect of CD10 antisense S-ODN was confirmed in the in vitro and animal models. Particularly, CD10 antisense S-ODN showed the preventive and therapeutic effects on liver metastasis in the mouse model. Further understanding of the biology of CD10 provides more effective application of CD10 targeting to clinics of CRCs.

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References


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