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Cleavage of carcinoembryonic antigen induces metastatic potential in colorectal carcinoma

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Abstract

Carcinoembryonic antigen (CEA), a widely used tumor marker, is attached by a glycosylphosphatidylinositol (GPI) anchor motif to the cell membrane. Recent study suggested that membrane-bound CEA might be cleaved by glycosylphosphatidylinositol-phospholipase D (GPI-PLD). We studied the effect of GPI-PLD on the cleavage of CEA to elucidate the implication for metastatic potential in colorectal carcinoma cells. CEA amount of conditioned medium was changed by suramin and phenanthroline (activator and inhibitor of GPI-PLD) only in SW620 and SW837 which expressed both CEA and GPI-PLD mRNA. Suramin treatment also augmented migratory activity and decreased cell surface CEA expression in SW620 and SW837. Furthermore, GPI-PLD knockdown cells using GPI-PLD-specific siRNA in SW620 and SW837 showed decreased CEA secretion from cell membrane and the migration activity, increased membrane-bound CEA amount. Splenic injection of SW620 and SW837 induced marked hepatic metastases in nude mice. These results suggest that membrane-bound CEA is cleaved by GPI-PLD and that this cleavage enhances the metastatic potential in colorectal carcinoma cells.

Keywords: CEA; GPI-PLD; Metastasis

Carcinoembryonic antigen (CEA) was discovered from the extract of colorectal cancer tissue as an oncofetal antigen and has been widely used as a human tumor marker. Biochemical and molecular analyses revealed that CEA [1] is a highly glycosylated cell surface protein which belongs to immunoglobulin superfamily adhesion molecules. CEA is attached to the cell surface membrane via a glycosylphosphatidylinositol (GPI) anchor and has been shown to function as homophilic or heterophilic cell adhesion molecules [2,3].

Although protein levels of CEA are significantly lower in adult colonic mucosa than in fetal one, they can become elevated when tumors arise in the stomach, small intestine, colon, rectum, pancreas, liver, breast, ovary, cervix, and lung [4,5]. Using sensitive immunoassays, CEA can be detected in the blood of cancer patients and is used as a diagnostic tool for several cancers. CEA, a membrane-bound glycoprotein by GPI anchor, should be cleaved by some enzyme to be detected as a soluble form in blood.

In vitro, at least two phospholipases can cleave the GPI-anchored cell surface protein. The first is a GPI-specific phospholipase C (GPI-PLC), although the enzyme is not specific for GPI and is not active towards mammalian GPI anchors. The second, GPI-phospholipase D (GPI-PLD), is abundantly present in serum and three human GPI-PLD isoforms have been identified in serum, liver, and pancreas [6]. These isoenzymes are extremely homologous to each other (>90%

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sequence identity) and are unrelated to other phospholipases. GPI-PLD is highly specific for the GPI anchor and does not cleave any other phospholipids. GPI-PLD enzyme activity is also expressed and secreted by a variety of cell lines including keratinocytes, bone marrow stromal cells, and many tumor cell lines [7,8].

In the present study, we examined the effect of GPI-PLD to cleave CEA in various colorectal carcinoma cell lines (SW620, SW837, Colo201, Colo320, and SW1736). And we also investigated whether the cleavage of CEA was concerned with cell migratory activity and metastatic potential.

Materials and methods

Cell culture. The human colorectal cancer cell lines (SW620, SW837, Colo201, Colo320, and SW1736) were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in Dulbecco's modified Eagle's medium (Nihonseiyaku Kogyo, Tokyo, Japan) supplemented with 10% fetal calf serum (FCS) (Daiichi Pure Chemicals, Tokyo, Japan), 50 U/ml penicillin, and 50 μg/ml streptomycin (Life Technologies, Gaithersburg, MD, USA) at 37 °C in a humidified atmosphere of 5% CO_2.

Total RNA isolation and RT-PCR assay. Total RNA was isolated from the cultured cells using TRIZOL regent (Invitrogen Life Technologies, Carlsbad, CA, USA). One microliter of total RNA was used for the synthesis of single-strand complementary DNA (cDNA) with oligo(dT) primer and reverse transcriptase (Promega, Madison, WI, USA) in a total volume of 20 μl, and then 1 μl was used as a template for polymerase chain reaction (PCR).

Amplification of specific DNA fragments was accomplished by adding 1 μg cDNA pool to a PCR mixture that contained 0.2 mM dNTPs, 1 μM each of 5′- and 3′-specific primers, 5 μl of 10× reaction buffer, and 1 μl of Advantage cDNA Polymerase Mix (Clontec Laboratories, Palo Alto, CA, USA) in a total volume of 50 μl. The thermal cycler profile consisted of an initial denaturation at 94 °C for 5 min, followed by 35 cycles with a 30 s denaturation at 94 °C, a 30 s annealing of primers at 60 °C, and a 30 s extension at 72 °C. The amplified products were separated on 1.5% agarose gel. The primer sequences and amplified product sizes were as follows: CEA (forward) 5′-TCTGCATCTGGAACTTCTCC-3′ and (reverse) 5′-TCTC TGCTTGTCTTGG-3′ (571 bp); GPI-PLD (forward) 5′-GGCGGAG CCACTCGGATGTACG-3′ and (reverse) 5′-AGCTCCTTCCAGCG GCAATGACG-3′ (370 bp); and β-actin (forward) 5′-TTGCTATC CAGGCTGTGCTAT-3′ and (reverse) 5′-CCTTAATGTCACGCA CGATTT-3′ (240 bp).

CEA ELISA. The concentration of CEA in the conditioned medium of the colon cancer cells was measured using ELISA kits (Hope Laboratories, Belmont, CA, USA). These cells were plated in a 24-well plate in a medium containing 10% FCS. When the cells became subconfluent, the medium was replaced with a serum-free medium. The cells were then incubated with 10 mM suramin or 1 mM of 1,10-phenanthroline (Wako Pure Chemical Industries, Osaka, Japan) for 48 h. To measure the CEA concentration, 50 μl conditioned media were put into each well of 96-well ELISA plate at room temperature. After the incubation for 1 h, incubation mixtures were removed and washed five times with phosphate-buffered saline solution (PBS). Tetramethylbenzidine (TMB) substrate of 100 μl was added to each well and incubated for 20 min. To stop the reaction, 100 μl of 2 N HCl was added to each well. The optical density was measured at 450 nm with microplate reader. Results were normalized for the number of the cells and evaluated as nanograms of CEA/10^6 cells/48 h.

Migration assay. Cells were collected by using a scraper and suspended with serum-free medium after the cells had been washed with the same medium. Migration of colon carcinoma cells was assessed by modified Boyden chamber assay. Eight-micrometer pore polycarbonate membrane was placed in a 24-well plate. DMEM supplemented with 10% FCS was used as a chemoattractant into the lower wells. Untreated or suramin-treated cells (5×10^5) were plated into the upper chamber and then allowed to migrate through the membrane at 37 °C in an atmosphere of 5% CO_2. After 3 h, cells that had not migrated through the membrane were scraped off with a cotton swab, and the membrane filter was fixed with 10% formalin and then stained with hematoxylin and eosin solution. The number of cells that had migrated to the lower side through the pores was counted with a microscope. Ten fields (200×) per chamber were counted, and the average numbers were determined from assays with three chambers.

Flow cytometry. Colon carcinoma cells were collected and resuspended in FACS medium (DMEM supplemented with 1% FCS and 0.03% NaN_3) to reach a final concentration of 5×10^5 cells/ml. The cells were incubated for 30 min on ice with the CEA mouse monocloned antibody (Sigma, Saint Louis, Missouri, USA) at 1:50 in FACS medium. After incubation, the cells were washed twice with FACS medium and incubated for 30 min on ice with anti-mouse IgG rabbit antibody conjugated with FITC (Sigma, Saint Louis, Missouri, USA) at 1:500 in FACS medium. They were washed twice with FACS medium and resuspended, as noted above. The stained cells were analyzed on an EPICS XL System (Beckman Coulter, Fullerton, CA, USA).

Gene knockdown using siRNA. Short interfering RNA that specifically targeted the GPI-PLD mRNA was used to reduce the gene expression [9]. We selected SW620 and SW837 cells as the representative cell lines for siRNA treatment because those cell lines expressed both CEA and GPI-PLD mRNA. The siRNA was generated by chemical synthesis (Invitrogen Life Technologies, Carlsbad, CA, USA). Cells (10×10^3) were grown on 24-well plates and were transfected with siRNA using an oligofectamine reagent (Invitrogen Life Technologies). The target sequences were 5′-GAGAAGACGGCCGA GTATATGTATA-3′ (GPI-PLD) and 5′-GAGAGGCCGGCGAAT ATGTTAAATA-3′ (GPI-PLD control). The sequences of target siRNA and control siRNA were derived from the GPI-PLD coding region, but the control siRNA did not have any effect on GPI-PLD expression. Blast analysis (http:www.ncbi.nlm.nih.gov/BLAST/) did not reveal overlapping regions between both target sequences and other human genes.

The amount of CEA in conditioned medium, migratory ability, and cell membrane CEA expression were examined also in GPI-PLD knockdown cells.

Liver metastasis experiment. In vivo liver metastasis capability of each colorectal cancer cell was evaluated in athymic nude mice ($n = 4$/each cell line). Four-week-old female BALB/c nude mice were purchased from Oriental Yeast, Tokyo, Japan, and maintained under specific pathogen-free conditions in facilities. The mice were housed in stainless-steel mesh cages under controlled conditions of temperature (23 ± 3°C) and relative humidity (50 ± 20%), with 10–15 air changes per hour and light for 12 h per day. The animals were allowed access to food and tap water ad libitum throughout the acclimatization and experimental periods. All of the animal experiments were approved by the Institutional Animal Care and Use Committee of Osaka University Graduate School of Medicine and Health Science before the experiments were started. A small left abdominal incision was made under sterile conditions and spleen was exteriorized. Viable 10^6 tumor cells in 0.05 ml of serum-free medium were injected into the spleen by means of a sterile tuberculin syringe and a 27-gauge needle. During the injection, care was taken to maintain uniform cell suspensions and to avoid injecting clumped cells. One minute after the injection splenectomy was performed. The abdomen was closed with nylon sutures, and the animals were returned to their cages. After 4 weeks, the animals

were euthanized and the abdominal organs, the thorax, and brain were examined for the presence of macroscopic and microscopic metastasis.

Statistics. The data were calculated as mean values and SDs. The statistical significance was tested by Student's *t* test.

Results

CEA and GPI-PLD mRNA expression

The expression of mRNA of CEA and GPI-PLD in five colorectal cancer cells was examined by RT-PCR which showed the 571- and 370-bp bands corresponding to CEA and GPI-PLD, respectively (Fig. 1). Five colorectal cancer cells revealed different expression patterns for CEA and GPI-PLD; SW620 and SW837 cells expressed both CEA and GPI-PLD, while neither CEA nor GPI-PLD was detected in SW1736 cells. Colo201 and Colo320 did not express both; the former expressed only CEA mRNA while the latter expressed only GPI-PLD.

Effects of GPI-PLD activity on the cleavage of CEA

We next examined the effect of GPI-PLD on the cleavage of CEA. We used the suramin or 1,10-phenanthroline which is an activator or inhibitor of GPI-PLD, respectively [10]. In SW620 and SW837 cells, suramin treatment enhanced the release of CEA about 3- to 4-fold over the untreated cell in conditioned medium ($p < 0.05$), on the other hand, phenanthroline reduced the CEA secretion ($p < 0.05$). The CEA secretion of Colo201 was slightly increased in conditioned medium, but statistical significance was not observed. The CEA secretion was scarcely detected in the conditioned medium of Colo320 and SW1736, and suramin or phenanthroline treatment did not change CEA secretion in these cells (Fig. 2).

Effects of GPI-PLD activity on cell migration

We also studied the effect of CEA release by GPI-PLD on cell migration of colon carcinoma cell lines using modified Boyden chamber assay, because CEA is a homophilic intracellular adhesion molecule which might regulate cell migratory ability. The marked migration activity was observed in SW620 cells and SW837 cells with suramin treatment ($p < 0.0001$; Fig. 3). Colo320 cells, Colo201 cells, and SW1736 cells had lower migration ability and no increased activity was induced by suramin in these cells.

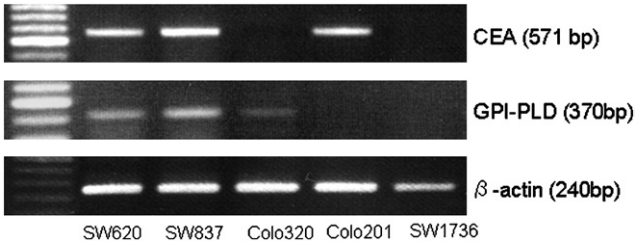

Fig. 1. CEA and GPI-PLD mRNA expression. The mRNA expression of both CEA and GPI-PLD was examined by RT-PCR method in colon carcinoma cell lines, SW620, SW837, Colo320, Colo201, and SW1736. The products on the 571- and 370-bp band correspond to CEA and GPI-PLD, respectively.

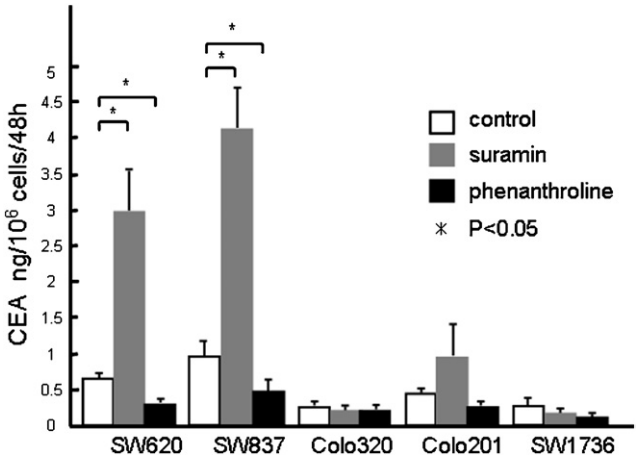

Fig. 2. Effects of GPI-PLD activity on the secretion of CEA. The CEA concentration of conditioned medium in colon carcinoma cell line was examined by ELISA. The secretion of CEA was induced by the suramin treatment, and, on the other hand, inhibited by phenanthroline in SW620 and SW837 cells. Values represent means ± SD of triplicate cultures. $*p < 0.05$. □, untreated cells; ■, suramin-treated cells; ■, phenanthroline-treated cells.

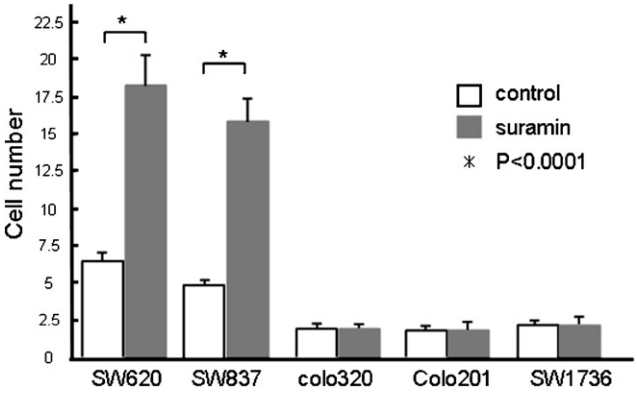

Fig. 3. Effect of CEA cleavage on the migration of colon carcinoma cells. Migration activity was assessed by modified Boyden chamber assay. With untreated or suramin-treated colon carcinoma cell lines were plated into the upper camber and then allowed to migrate though the membrane for 3 h. The number of migrated cells is shown on the vertical axis. Data are expressed as mean cell counts ($n = 3$); bars, ±SD. $*p < 0.0001$. □, untreated cells; ■, suramin-treated cells.

Membrane-bound CEA expression by flow cytometry

CEA expression on the cell membrane in colorectal carcinoma cells was examined by flow cytometry.

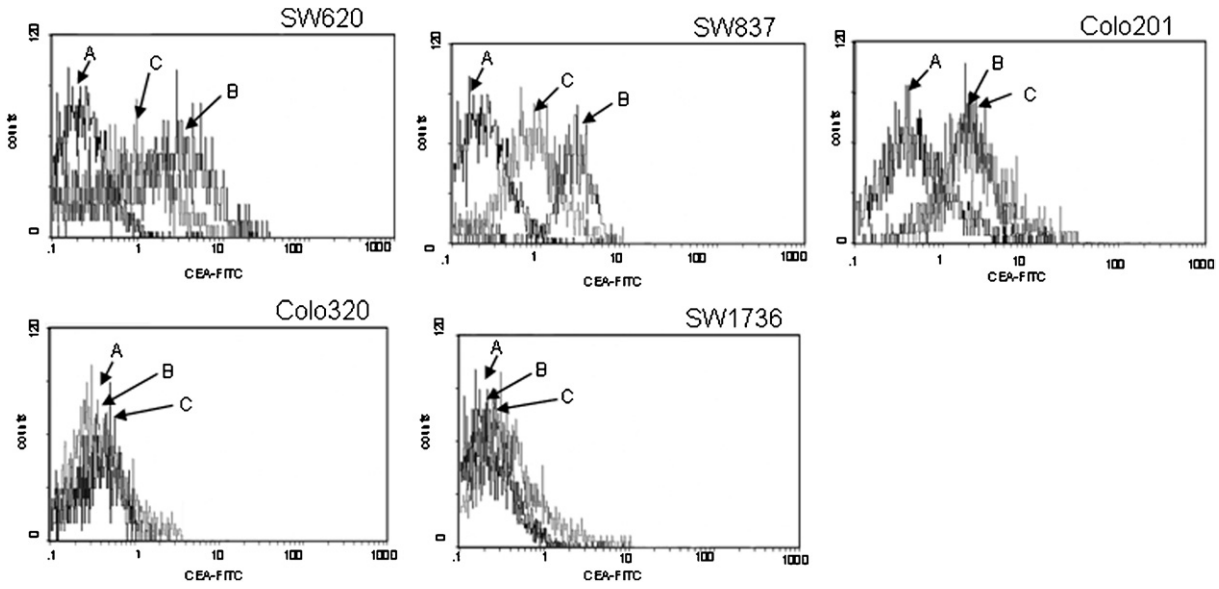

Fig. 4. Effects of GPI-PLD activity on the expression levels of membrane-bound CEA in colon carcinoma cells by flow cytometric analysis. Suramin treatment resulted in decreased expression level of membrane-bound CEA only SW620 and SW 837 cells. A, background staining with isotype control Ab. B, expression of CEA in suramin untreated cells. C, expression of CEA in suramin-treated cells.

Although CEA expression was observed on the cell surface of SW620, SW837, and Colo201, a reduction of membrane-bound CEA expression was detected only in SW620 and SW837 with suramin treatment. Suramin treatment did not change the surface CEA expression in another cell lines (see Fig. 4).

Effect of GPI-PLD knockdown

In order to elucidate the functional role of GPI-PLD in colon carcinoma cell lines, we studied the effect of decreased GPI-PLD level using GPI-PLD-specific siRNA to reduce its expression in the SW620 and SW837 with higher expression of GPI-PLD by RT-PCR (Fig. 1). As shown in Fig. 5A GPI-PLD mRNA expression was significantly reduced 48 h after treatment of GPI-PLD siRNA in both cell lines while control siRNA did not decrease it. The CEA secretion in conditioned medium was also reduced by GPI-PLD siRNA treatment (Fig. 5B). Furthermore, GPI-PLD siRNA treatment caused reduced cell migratory activity (Fig. 6A) and increased cell surface CEA expression (Fig. 6B) compared with control siRNA treatment.

Liver metastasis after splenic injection

Four weeks after splenic injection of colorectal carcinoma cells, mice were sacrificed and every organ was examined for metastases. Four of four mice injected with SW837 and three of four mice injected with SW620 developed liver metastases. On the other hand, no liver metastasis was detected in any mice with Colo320, Colo201 or SW1736 cell inoculation. All the liver metastatic tumors were confirmed histologically to be secondary colorectal carcinoma. No other metastasis was found in any mice.

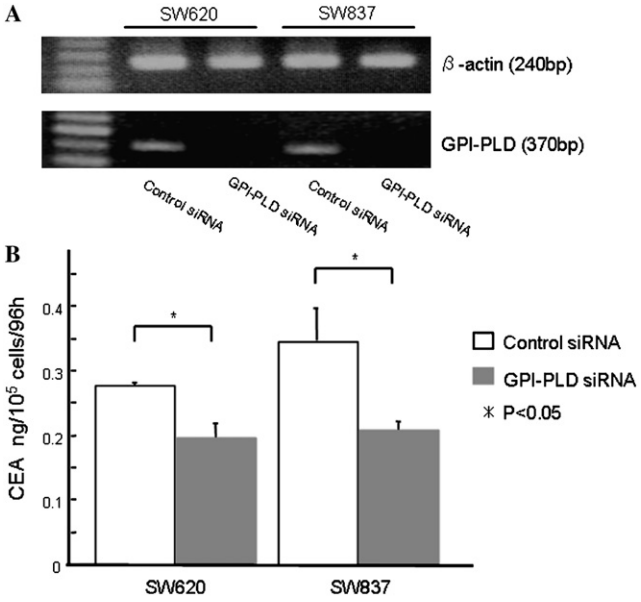

Fig. 5. Effect of GPI-PLD siRNA treatment on CEA secretion. (A) Decreased mRNA expression by GPI-PLD siRNA. SW620 and SW837 cells were plated at an initial density 10×10^3 cells and 24 h after plating were transfected with 20 μg GPI-PLD siRNA . Forty-eight hours post-transfection cells were collected, and RNA was extracted and the mRNA expression was analyzed by RT-PCR. (B) CEA concentration in the conditioned medium. Effect of GPI-PLD knockdown on CEA secretion was examined by CEA ELISA in conditioned medium of SW620 and SW837 with siRNA transfected after incubation for 96 h. Values represent means ± SD. ($n = 3$). *$p < 0.01$. □, control siRNA cells; ■, GPI-PLD siRNA cells.

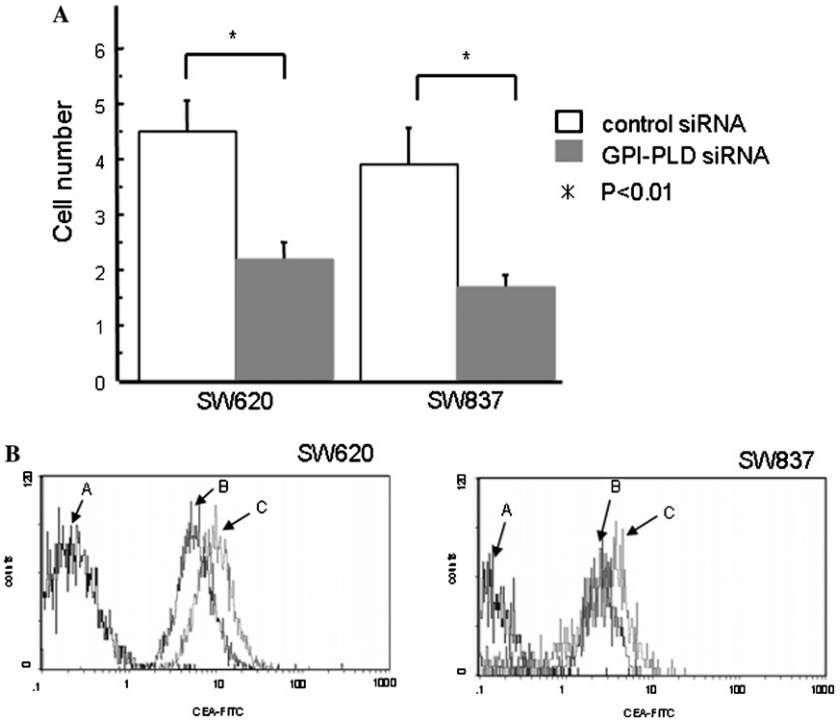

Fig. 6. Effects of GPI-PLD siRNA treatment on cell migration activity (A) and cell surface CEA expression (B). (A) Migration activity was examined in SW620 and SW837 with transfection control siRNA or GPI-PLD siRNA. The number of migrated cells is shown on the vertical axis. Values represent means ± SD. ($n = 3$), *$p < 0.01$. □, control siRNA cells; ■, GPI-PLD siRNA cells. (B) The cell surface CEA expression was measured by flow cytometry. A, background staining with isotype control Ab alone. B, expression of CEA in control siRNA cells. C, expression of CEA in GPI-PLD siRNA cells.

Discussion

CEA, GPI-anchored protein, is a member of cell surface glycoproteins of immunoglobulin superfamily [11] and soluble form of CEA, after cleavage from the cell membrane, is measured in the serum of the patient with cancer as widely used tumor marker. Because CEA is overexpressed in more than 50% of human cancers [12], it would seem experimentally and clinically important to elucidate a mechanism of releasing CEA from the cell surface. Recent evidence indicates that GPI-PLD, specific for GPI-anchor glycoproteins, is involved in the release of CEA from the membrane. When GPI-PLD and GPI-anchored proteins are co-expressed in the same cells, GPI-anchored proteins are released from the cell membrane by GPI-PLD [13,14]. Placental alkaline phosphatase (PLAP) is a well-characterized GRI-anchored protein. Tsujioka et al. [15] examined the involvement of GPI-PLD in the release of PLAP in HeLa cells that had been transfected with the GPI-PLD cDNA. When PLAP was expressed alone, the newly synthesized PLAP remained associated with the cell surface and was not secreted into the medium. In contrast, when PLAP was co-expressed with GPI-PLD, a substantial amount of PLAP was detected into the medium, suggesting that the release of PLAP is caused by the exogenously introduced GPI-PLD.

In the present study, we examined the mRNA expression of CEA and GPI-PLD in various colorectal carcinoma cell lines and found that SW620 and SW837 cells expressed both, Colo201 cells expressed only CEA, Colo320 cells expressed only GPI-PLD, and SW1736 cells expressed neither. And we showed that the secretion of CEA in the conditioned medium was increased by suramin (GPI-PLD activator) and decreased by phenanthroline (GPI-PLD inhibitor) only in both the CEA and GPI-PLD expressing cells, suggesting that CEA could be cleaved by GPI-PLD. We examined the amount of secretion of alkaline phosphatase (ALP) in order to confirm that GPI-PLD activity is controlled by both reagents' treatment. The ALP secretion was changed by both reagents' treatment in colon carcinoma cell lines (data not shown). We generated the GPI-PLD knockdown cells by using the GPI-PLD-specific siRNA. The GPI-PLD knockdown cells decreased the CEA cleavage from cell membrane in conditioned medium. These results suggest that CEA secretion can be induced in the cleavage of cell-bound CEA by GPI-PLD in colon carcinoma cell lines.

CEA is a homotypic intercellular adhesion molecule and might regulate cell movement by its cell–cell binding activity. The cleavage of CEA could induce downregulation of the cell adhesive ability, and cell–cell binding becomes loose and the cell movement activity is increased as a result as seen in the downregulation of E-cadherin

by sugar chain modification [16]. We examined the migration activity and the cell membrane CEA expression in colorectal carcinoma cells with suramin treatment or GPI-PLD siRNA. The increased migration activity was observed only in SW620 and SW837 cells with suramin treatment in which CEA secretion was significantly enhanced. Furthermore, the cell surface CEA expression was reduced by suramin treatment. On the other hand, GPI-PLD knockdown cells showed the reduction of migration activity and an increase in the cell membrane CEA expression. These results suggested that the cell surface CEA cleavage by GPI-PLD promoted cell migration. We tested in vivo metastatic ability of each cell line in nude mice. Since colorectal cancer usually induces liver metastasis via portal vein, splenic injection model is frequently used in which injected cancer cells go into splenic sinusoid and flow to liver through portal vein. As a result only SW837 and SW620 cells developed marked liver metastases after splenic injection in the nude mice. Taken together when both CEA and GPI-PLD are expressed in colorectal carcinoma cells, high CEA secretion is not only observed in those cells but also those cells have more migratory activity in vitro and develop liver metastasis in vivo.

Clinically the colon cancer patients with high serum CEA concentration have been known to have poor prognoses. CEA has been experimentally implicated in the development of hepatic metastasis [17,18]. CEA is primarily recognized and endocytosed by an 80 kDa receptor of both human and rodent Kupffer cells [19]. The activation of 80 kDa rat Kupffer cell receptor by CEA induces tyrosine phosphorylation which is associated with the production of cytokines TNF-α and IL-1α. The cytokines, particularly IL-1α and TNF-α, produced by the Kupffer cells have the potential to affect not only the growth characteristics of the invading tumor cells but also the biological responses of endothelial and other cells in the hepatic sinusoid and may have substantial relevance to the formation of hepatic metastasis from colorectal cancer [20]. The intravenous injection of CEA in athymic nude mice, prior to the intrasplenic injection of colorectal carcinoma cell lines with a low metastatic potential, has been reported to enhance hepatic metastasis [21]. In addition, human colon carcinoma cell lines have also been shown to acquire a highly metastatic potential when transfected with the cDNA coding for CEA [22,23]. These data suggested that CEA play a role in the formation of hepatic metastasis. It is considered that the circulating CEA cleaved by GPI-PLD might enhance the formation of hepatic metastasis.

Cleavage of GPI-anchor protein by the GPI-PLD has the potential to generate the second messengers (phosphatidic acid and diacylglycerol). Phosphatidic acid can elevate intracellular Ca^{2+} levels and induce mitogenesis in certain cell types [24,25]. DAG produced in response to various stimulations is known to activate protein kinase C (PKC) which plays a key regulatory role in a variety of cellular functions including cell growth, differentiation, gene expression, and hormone action [15]. Therefore, GPI-PLD might regulate the general biological function of GPI-anchored protein including CEA by generating soluble isoforms with distinct biological activities. These findings suggest that the CEA secretion plays an important role to control the various signals in the mechanism of metastasis of colon carcinomacells.

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