Detection of Increased Fecal Carcinoembryonic Antigen and Its Characterization as a Membrane-bound Form in Colorectal Carcinoma and Other Gastrointestinal Disorders

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The levels and character of carcinoembryonic antigen (CEA) in feces were investigated by sandwich radioimmunoassay using anti-CEA monoclonal antibodies NCC-CO-411 and NCC-CO-432. Mean CEA concentration was significantly higher ($P < 0.001$) in the feces from patients with colorectal carcinoma and other gastrointestinal disorders as compared to normal adults. More than 90% of the fecal CEA was trapped by a 0.22 μ m membrane filter and solubilized by treatment with 1% Triton X-100 or phosphatidyl-inositol specific phospholipase C. In hydrophobic chromatography, most of the fecal CEA was eluted at the lowest $(\text{NH}_4)_2\text{SO}_4$ concentration while serum CEA appeared in the more hydrophilic fractions. These results suggest that the majority of CEA exists in feces as an amphiphilic molecule or a membrane-bound form. The increase of fecal CEA may reflect the destruction and abrasion of epithelial cells in various gastrointestinal disorders.

Key words: Monoclonal antibody — Carcinoembryonic antigen — Phosphatidyl inositol-linked membrane protein — Phosphatidyl-inositol specific phospholipase C

Carcinoembryonic antigen (CEA) is a clinically useful tumor marker in serodiagnosis of carcinoma.¹⁾ Originally CEA was thought to be a specific marker for colorectal carcinoma.^{2,3)} However, it was found that elevation of serum CEA is not specific to colorectal carcinoma, but also occurs in patients with carcinomas originated from other organs and miscellaneous benign disorders. In addition, serum CEA level remained within normal limits in most of the patients with early colorectal carcinoma. Thus, the determination of serum CEA was thought unavailable for mass screening of colorectal carcinoma.¹⁾ In applying CEA as a screening marker for colorectal carcinoma, determining the CEA excreted in the feces seems a more promising approach, since the feces may contain CEA directly excreted from tumor tissue. However, feces contain several CEA-related substances such as normal fecal cross-reacting antigen (NFCA), normal fecal antigen-1 (NFA-1), normal fecal antigen-2 (NFA-2) and biliary glycoprotein-1 (BGP-1),⁴⁻⁸⁾ so the determination of fecal CEA as an aid in diagnosing colorectal carcinoma has been thought to be unhelpful, although a trial was reported by using a commercial CEA-RIA kit with a conventional antiserum.⁹⁾

In this paper, we present a newly established sandwich radioimmunoassay (RIA) for fecal CEA using

anti-CEA monoclonal antibodies, NCC-CO-411 and NCC-CO-432, and we describe the characterization of the fecal CEA as a membrane-bound molecule.

The generation, characterization and reactivity of the murine monoclonal antibodies, NCC-CO-411(IgG1) and NCC-CO-432(IgG1), have been described elsewhere.¹⁰⁾ In brief, both antibodies were screened for immunohistological reactivity with colon carcinoma tissues. NCC-CO-411 was slightly reactive with granulocytes and spleen, thus it was thought to react with nonspecific crossreacting antigen (NCA), which is a CEA-related substance.¹¹⁾ However, neither of these antibodies reacted with purified NCA and BGP-1 by enzyme-linked immunosorbent assay (ELISA). Neither of the antibodies reacted with CEA in immunoblotting after transfer from polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) and 2-mercaptoethanol (2ME), so they were thought to recognize some 3-dimensional conformation of the CEA molecule.¹⁰⁾ These antibodies were purified from the ascitic fluid of BALB/c mice inoculated with hybridoma cells, using protein A affinity chromatography. The iodination of NCC-CO-432 and conjugation of NCC-CO-411 to polystyrene beads was performed as previously described.^{12,13)} The specific activity of ¹²⁵I-labeled NCC-CO-432 antibody ranged from 4 to 4.5 Ci/g. Purified CEA from ascitic fluid from a patient with colon carci-

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noma was used as an assay standard. Concentrations of this standard were adjusted with the purified CEA commercially available from DAKO Corp. (CA). Stool samples (20 mg) from normal adults, and patients with colorectal carcinoma and other gastrointestinal disorders were measured and dissolved in 5 ml of 0.1 M, pH 7.4 phosphate-buffered saline (PBS) containing 0.15 M NaCl and 0.2% sodium azide. After centrifugation at 3,000 rpm for 15 min, the supernatants were recovered and assayed. Experimental samples were assayed in duplicate with a "forward sandwich" RIA.¹²⁾ We mixed in a reaction tray 50 μ l of sample, standards, or culture medium of colon carcinoma cell line C-1 as a positive control with 200 μ l of buffer (PBS containing 5% normal equine serum and 0.1% sodium azide). One antibody-coated bead was added to each reaction well and the tray was incubated at 25°C for 2 h. Beads were washed three times with distilled water, then 200,000 cpm of ¹²⁵I-labeled NCC-CO-432 antibody in 200 μ l of the buffer was added. After incubation of the samples at 25°C for 2 h, the beads were washed three times and their radioactivity was counted with a gamma counter. A standard curve was

constructed by plotting standard antigen concentrations per ml vs. cpm. Experimental results were converted into CEA concentrations per ml by comparison with a standard curve.

The fecal CEA levels were 0.04 ± 0.03 μ g/ml (mean $\pm$ SD) in normal adults (n=15), 0.46 ± 0.51 μ g/ml in the patients with primary colorectal carcinoma (n=24), and 0.17 ± 0.19 μ g/ml in the patients with benign gastrointestinal disorders (n=55), and the differences between the respective groups are significant at $P < 0.001$ (Fig. 1). The cut-off value was determined as 0.26 μ g/ml to give the highest diagnostic efficiency, i.e., to provide optimum sensitivity and specificity. The positive rates in patients with colorectal carcinoma were 50.0% (12/24) in the primary cases including 50.0% (3/6) in Dukes A, 62.5% (5/8) in Dukes B, 50.0% (3/6) in Dukes C and 33.3% (1/3) in Dukes D. The positivity was relatively uniform regardless of the stage, and the fecal CEA value decreased to under the cut-off value after curative operation. In the patients with recurrent colorectal carcinoma, 50.0% (3/6) had positive values and all of the positive cases had local recurrence in colorectum, so that the

Fig. 1. Fecal CEA concentrations in normal adults, patients with colorectal carcinoma and benign gastrointestinal disorders. The concentrations of fecal CEA were significantly increased in the patients with colorectal carcinoma (0.46 ± 0.51 μ g/ml, $P < 0.001$) and in the patients with benign gastrointestinal disorders (0.17 ± 0.19 μ g/ml, $P < 0.001$) as compared to normal adults (0.04 ± 0.03 μ g/ml). Each value is the mean $\pm$ SD. The significance of differences in antigen concentration was evaluated by using the unpaired *t*-test.

Table I. Positive Rates of Fecal CEA in Patients with Various Gastrointestinal Disorders

	No. tested	No. cases positive	Positive rates (%)
Malignant disorders			
Carcinoma of colorectum	30	15	50.0
Dukes A	6	3	50.0
Dukes B	8	5	62.5
Dukes C	6	3	50.0
Dukes D	3	1	33.3
recurrent	6	3	50.0
Carcinoma of stomach	14	5	35.7
primary	8	2	25.0
recurrent	6	3	50.0
Benign gastrointestinal disorders			
stomach and duodenal ulcer	7	2	28.6
cholelithiasis and choledocholithiasis	15	2	13.3
liver cirrhosis	5	0	0.0
inflammatory bowel disease	10	6	60.0
(with melena)	7	5	71.4
colonic polyp	6	3	50.0
diverticulosis	3	1	33.3
pile and anal fistula	6	0	0.0
appendicitis	2	0	0.0
Normal adults	15	0	0.0

feces were thought to have passed through the cancerous lesion. In 35.7% (5/14) of the patients with gastric carcinoma, positive values were also observed. However, the fecal CEA also increased in patients with benign gastrointestinal disorders such as gastroduodenal ulcer, cholelithiasis, choledocholithiasis, inflammatory bowel disease (IBD), colonic polyp and diverticulosis. Particularly, in the patients with IBD accompanied with massive bleeding, 71.4% (5/7) had positive antigen values (Table I).

To characterize the fecal CEA defined by this assay, 10 samples which had elevated CEA were filtered using a 0.22 μ m pore-sized membrane filter (Millipore Corp., MA), and the filtrates were assayed. In 9 out of 10 samples, more than 90.0% of the antigenic activity was trapped by the filter, and this impermeability disappeared after solubilizing the sample in the presence of 1% Triton X-100 (Table II). These results suggested that the antigen existed in the feces as an aggregated protein or in a macromolecular form. Recently, CEA was shown to be a phosphatidyl inositol-linked membrane protein which was readily released and solubilized by the action

Table II. Filtration of the Fecal Samples Using a 0.22 μ m Membrane-filter

	Fecal CEA		Fecal CEA in the filtrate	
	before filtration (μ g/ml)	no detergent (μ g/ml)	with 1% TritonX-100 (μ g/ml)	
No. 1	0.81	0.01 (1.2) ^{a)}	0.80 (100.0)	
No. 2	0.92	0.02 (2.2)	0.91 (98.9)	
No. 3	1.00	0.00 (0.0)	0.75 (75.0)	
No. 4	0.45	0.02 (4.4)	0.45 (100.0)	
No. 5	0.33	0.02 (6.1)	0.32 (97.0)	
No. 6	0.42	0.15 (35.7)	0.41 (97.6)	
No. 7	0.25	0.01 (4.0)	0.23 (92.0)	
No. 8	0.38	0.03 (7.9)	0.29 (76.3)	
No. 9	0.37	0.02 (5.4)	0.30 (81.1)	
No. 10	0.24	0.00 (0.0)	0.24 (100.0)	
	$0.52 \pm 0.27^b)$	0.03 ± 0.04 (5.8)	0.47 ± 0.24 (90.4)	

a) The value in the parenthesis is the percentage with respect to the value before filtration.

b) Mean $\pm$ standard deviation (SD).

Table III. Treatment of Insoluble Fecal Fraction with PI-PLC and Triton X-100

	Control (ng/ml)	PI-PLC treated (ng/ml)
Sup. 1 ^{a)}	766.3 (93.2) ^{e)}	1015.0 (88.4)
Sup. 2 ^{b)}	5.4 (0.7)	2.5 (0.2)
Sup. 3 ^{c)}	3.6 (0.4)	102.3 (8.9)
Solute by Triton X-100 ^{d)}	46.7 (5.7)	28.9 (2.5)

a) Supernatant obtained after the first centrifugation at 20,000 rpm, 20 min.

b) Supernatant obtained from the precipitate of the first centrifugation, which was resuspended and separated by a second centrifugation at 20,000 rpm, 20 min.

c) Supernatant obtained from the precipitate of the second centrifugation, which was resuspended, incubated in 5 mM pH 7.4 phosphate buffer with 0.2 U/ml PI-PLC (PI-PLC treated) or without PI-PLC (control) at 37°C, 2 h and separated by a third centrifugation at 20,000 rpm, 20 min.

d) Solute obtained by adding 1% Triton X-100 to the precipitate of the third centrifugation.

e) The value in the parenthesis is the percentage with respect to the value of the total amount.

of phosphatidyl-inositol specific phospholipase C (PI-PLC).^{14,15)} Twenty milligrams of feces from a patient with colon carcinoma was suspended in 5 ml of 5 mM phosphate buffer (PB), pH 7.4 and centrifuged at 3,000 rpm for 15 min. Subsequently the supernatant was centrifuged at 20,000 rpm for 20 min to remove the soluble fraction and the supernatant was assayed. Then the precipitate of the first centrifugation at 20,000 rpm, 20 min was suspended in 5 ml of PB and centrifuged at

20,000 rpm, 20 min again. The antigenic activity in the supernatant of the second centrifugation at 20,000 rpm, 20 min decreased to as low as 2.5 ng/ml and the majority of the soluble fraction was thought to have been removed. Then the precipitate of the second centrifugation was suspended in 5 ml of PB containing PI-PLC from *B. thuringiensis* at a concentration of 0.2 U/ml and incubated at 37°C for 2 h, according to the method described by Taguchi and Ikezawa.¹⁶⁾ The sample was recentrifuged at 20,000 rpm for 20 min and the supernatant of the third centrifugation was assayed. Finally, the precipitate of the third centrifugation was solubilized by adding 5 ml of PB containing 1% Triton X-100 and the antigenic activity in this solute was determined as the residual antigen (Table III). After the treatment with PI-PLC, the CEA value in the supernatant increased and this release of CEA was revealed to depend on the concentration of the added PI-PLC and the incubation time (Fig. 2). After the solubilization by 1% Triton X-100 or PI-PLC, the samples were chromatographed using the FPLC system with a Superose 6 column (Pharmacia, Sweden) and compared with the CEA standard purified by conventional perchloric acid extraction. It was shown that the solubilized CEA had a molecular weight of approximately 180 kDa on gel chromatography and was indistinguishable from the standard CEA (data not shown). However, the amount of the antigen released by PI-PLC was 8.9% of the total amount and 88.4% of the antigen existed in the supernatant fraction of the first centrifugation at 20,000 rpm, 20 min. The hydrophobicity of the fecal CEA contained in this supernatant frac-

Fig. 2. Release of fecal CEA from insoluble fraction of the feces after the treatment with PI-PLC. The insoluble fractions were incubated with PI-PLC at concentrations of 0 unit/ml (●), 0.025 unit/ml (○), 0.05 unit/ml (■), 0.1 unit/ml (□), 0.2 unit/ml (●).

tion was examined and compared with that of serum CEA in a patient with colorectal carcinoma by chromatography on a butyl-Toyopearl column (1.0×10.0 cm) equilibrated with 0.5 M (NH₄)₂SO₄ in 50 mM PB, pH 7.4. The major antigenic activity of the fecal CEA was eluted at the lowest concentration of the decreasing (NH₄)₂SO₄ gradient, while most of the serum CEA appeared in the more hydrophilic fractions (Fig. 3a and 3b). It was interesting that the elution pattern of these amphiphilic fecal CEA fractions had two major peaks and the second peak seemed to be identical with the minor amphiphilic fractions found in serum CEA. The appearance of these amphiphilic forms may be attributed to the linkage of some fatty acid residue to the CEA molecule. We consider that these results strongly suggest that CEA exists in the stools as an amphiphilic molecule, presumably as a membrane-bound form via a phosphatidylinositol link. It was not clear if such membrane-bound form also exists in the case of other CEA-related substances in the feces. Previous studies concerning CEA and related substances started from purification through physicochemical procedures such as perchloric acid extraction and subsequent gel filtration,^{2-6, 8, 9, 11)} but these

Fig. 3. Butyl-Toyopearl column chromatography of CEA from feces and serum. (a) Fecal sample after centrifugation of 20,000 rpm, for 20 min was applied to a butyl-Toyopearl column (1.0×10.0 cm), and eluted with 20 ml of a decreasing linear gradient from buffer A (0.5 M (NH₄)₂SO₄ in 50 mM phosphate buffer) to buffer B (distilled water) at a flow rate of 1 ml/min. (b) Serum from a carcinoma patient with elevated serum CEA was applied to the same column. The conditions for elution were the same as described in (a). —○—, antigenic activity of CEA; —, A280 nm; - - - - - , (NH₄)₂SO₄ concentration.

approaches may not necessarily reflect the nature of CEA *in vivo*. That is, the conventional CEA or related substances themselves may be denatured forms of the native CEA or their counterparts. It is assumed that the increase of such a membrane-bound form may be a useful marker in screening colorectal carcinoma and other gastrointestinal disorders in the mass population, since the increase of fecal CEA could be explained in terms of the quantitative increase of cellular destruction and abrasion from the gastrointestinal tract caused by several pathogenic changes such as tissue necrosis, severe inflammation and ulceration. Further investigations should be

pursued to clarify the significance of membrane-bound CEA in the feces as a screening marker for gastrointestinal disorders, particularly for colorectal carcinoma.

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