Acta Medica
Okayama

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Original Article

Improvements in the Measurement of Stool Decay-Accelerating Factor in the Detection of Colorectal Cancer

Shogen Ohya[a], Motowo Mizuno[a]*, Mikihiro Kawada[a], Junichirou Nasu[a],
Hiroyuki Okada[a], Hiroyuki Shimomura[a], Kazuhide Yamamoto[a],
Teizo Fujita[b], and Takao Tsuji[a]

[a]Department of Medicine and Medical Science, Okayama University Graduate School of
Medicine and Dentistry, Okayama 700-8558, Japan, and
[b]Department of Biochemistry, Fukushima Medical College, Fukushima 960-1295, Japan

We have previously developed an enzyme-linked immunosorbent assay (ELISA) to measure stool decay-accelerating factor (DAF) and found that stool DAF concentrations were significantly elevated in patients with colorectal cancer, suggesting that the measurement of stool DAF may be valuable for the detection of colorectal cancer. In order to refine the assay for the measurement of stool DAF, we investigated 1) effects of centrifugation of stool samples, 2) effects of detergents, and 3) adequate combination of various anti-DAF monoclonal antibodies for the ELISA system using only monoclonal antibodies. We found that high-speed centrifugation could be omitted and that only the removal of large undigested food residues by centrifugation of short duration in a low-speed benchtop microcentrifuge sufficed to adequately prepare the stool samples. Addition of 2 detergents, octyl β-glucoside and sodium deoxycholate, known to solubilize glycosyl-phosphatidylinositol-anchored proteins such as DAF, did not influence stool DAF values. By using 2 mouse anti-DAF monoclonal antibodies (clone 4F11 and 1C6), we were able to achieve a stable ELISA for the measurement of stool DAF using a uniform source of antibodies. The results should allow us to consistently apply the DAF assay for routine use in the detection of colorectal cancer.

Key words: decay-accelerating factor (DAF), colorectal cancer, enzyme-linked immunosorbent assay (ELISA), monoclonal antibodies.

For the screening of colorectal cancer, fecal occult blood (FOB) testing has been widely used. However, FOB is not specific to colorectal cancer, and the efficacy of FOB testing remains controversial [1-4]. Thus, the development of more reliable markers for colorectal cancer is important.

Decay-accelerating factor (DAF) is a membrane glyco-

protein which regulates complement activation by inhibiting the formation of C3/C5 convertases and promotes their catabolism [5, 6]. We have previously demonstrated enhanced expression of DAF in human colorectal cancer [7, 8]. In addition, we developed an enzyme-linked immunosorbent assay (ELISA) to measure DAF in stools and found that stool DAF concentrations were significantly elevated in patients with colorectal cancer [9]. In this study [9], we also showed that stool DAF was detected in a substantial portion of patients with colorectal cancer who had a negative FOB test and that

the combination of the stool DAF measurement with FOB testing significantly increased the sensitivity for the detection of colorectal cancer. These findings have suggested that the measurement of stool DAF may be valuable for the detection of colorectal cancer.

Previously, we studied the characterization of DAF released into the culture supernatant of HT-29 human colon cancer cells and found that released DAF was present in its membrane-bound form as well as in its soluble form [10]. Thus, in the measurement of DAF in stool samples, the preparation of stool samples by techniques such as centrifugation and the addition of detergents possibly influenced the resulting DAF measurements. In addition, in the original ELISA for stool DAF, we used a combination of mouse monoclonal and rabbit polyclonal anti-DAF antibodies [9]; the use of the polyclonal antibody could have been an obstacle to achieving a stable DAF assay; using a uniform source of antibodies was therefore indicated. In the present study, to refine the assay for the measurement of stool DAF, we investigated the following: 1) effects of centrifugation of stool samples, 2) effects of detergents, and 3) preferable combination of various anti-DAF monoclonal antibodies for the development of an ELISA system using only monoclonal antibodies to measure stool DAF.

Materials and Method

Preparations of stool specimens.   Spontaneous stool samples (1–5 g) were obtained from 10 patients with colorectal cancer (4 women and 6 men; 58–82 years old; mean age: 68 years) and from 8 healthy volunteers (2 women and 6 men; 45–72 years old; mean age: 58 years). All healthy participants had undergone total colonoscopic examination due to abdominal symptoms and/or screening for colorectal cancer; the healthy controls were found to have no colorectal disease. Informed consent was obtained from each patient.

In the original ELISA assay, the stools were weighed, suspended in an equal volume of phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA), 0.05% Tween 20 and 1 mM phenylmethylsulfonylfluoride (sample buffer), and centrifuged at 20,000 g for 15 min. Supernatants were collected and kept frozen at $-80\,^{\circ}C$ until use. To analyze the effects of centrifugation, stool samples were prepared without the use of high-speed centrifugation. Weighed stool samples were suspended in the sample buffer and

centrifuged in a low-speed benchtop microcentrifuge for 30 sec to remove large undigested food residues. Supernatants were collected and kept frozen as described above.

To examine effects of detergents, 2 detergents, octyl β-glucoside (Pierce, Rockford, IL, USA) and sodium deoxycholate (Sigma Chemical Co., St. Louis, MO, USA) were added to the sample buffer. Stools were suspended in each of the sample buffers containing octyl β-glucoside (60 mM) or sodium deoxycholate (9 mM) and were incubated at $4\,^{\circ}C$ for 30 min and centrifuged in a low-speed benchtop microcentrifuge for 30 sec. Supernatants were collected and kept frozen, as described above.

ELISA for the measurement of DAF.   For the analysis of the effects of centrifugation and detergents, the original ELISA system was prepared as described [9]. Briefly, the wells of microtiter plates (Nunc, Roskilde, Denmark) were coated with 1C6 mouse monoclonal anti-DAF antibody (IgG1$\varkappa$ isotype). Stool supernatants were added to the wells, and then rabbit polyclonal anti-DAF IgG was added. After washing, bound rabbit antibody was detected with horseradish peroxidase (HRP)-labeled goat F(ab')2 anti-rabbit IgG (TAGO, Inc., Burlingame, CA, USA) and 2,2'-azino-di-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) as substrate. Optical densities (OD) at 415 nm were measured on an automated ELISA plate reader. A calibration curve was obtained from several dilutions of known quantities of purified DAF, and the concentrations of stool DAF were calculated. Samples were analyzed in duplicate.

To refine the ELISA system using only monoclonal antibodies, we prepared, as described [11], 2 mouse monoclonal anti-DAF antibodies (clone 4F11 and 5B2) in addition to the 1C6 anti-DAF monoclonal antibody. All of these antibodies belong to the IgG1$\varkappa$ isotype and recognize different epitopes on the DAF molecule [11, 12]. The 1C6 antibody is directed to the active site on the DAF molecule, *i.e.*, short consensus repeat (SCR) 3, whereas the 4F11 and 5B2 antibodies recognize SCR 4 and SCR 2, respectively [12]. Biotin-labeled 1C6 and 4F11 anti-DAF antibodies were prepared as described [13, 14]. Human DAF was purified from pooled human erythrocyte stroma as described [5, 11].

To determine the most adequate combination of these anti-DAF monoclonal antibodies, the following combination was examined: 4F11 or 5B2 antibody on the ELISA plate with biotin-labeled 1C6 antibody as a second antibody, and 1C6 or 5B2 antibody on the ELISA plate with

biotin-labeled 4F11 antibody as a second antibody. Various amounts of purified DAF were added to each microtiter plate coated with 100 μl of one of the three anti-DAF monoclonal antibodies (10 μg/ml) and incubated at 4 °C overnight. After washing with PBS containing 0.05% Tween 20, 100 μl of either biotin-labeled 1C6 (500 ng/ml) or biotin-labeled 4F11 was added to the wells. After incubation at room temperature for 2 h and washing, bound biotin-labeled antibodies were detected with HRP-labeled streptavidin (Chemicon International, Inc., Temecula, CA, USA) and ABTS as the substrate. OD at 415 nm were measured as described above. After we determined the combination of the monoclonal antibodies, we defined the dynamic range for the new ELISA system by measuring various amounts of purified DAF; we then evaluated the relationship between DAF as determined by the new and the previous ELISAs using Pearson's correlation coefficient.

Results

Effects of centrifugation and detergents in the preparation of stool samples.   Although a few of the stool samples treated by low-speed centrifugation yielded slightly higher values than did those with high-speed centrifugation, stool DAF amounts in the samples prepared with high-speed and low-speed centrifugation did not differ remarkably (Table 1). Addition of either octyl β-glucoside or sodium deoxycholate detergent to the sample buffer did not apparently influence DAF values (Table 2).

New ELISA using only monoclonal anti-DAF antibodies.   Among the combinations of monoclonal antibodies examined, the combination of 4F11 and 1C6 antibodies yielded higher OD values than did those with any combination including 5B2 antibody (Fig. 1). OD values did not differ remarkably when 4F11 antibody was present on the ELISA plate with biotin-labeled 1C6 antibody as a second antibody or when 1C6 antibody was present on the ELISA plate with biotin-labeled 4F11 antibody as a second antibody. We therefore arbitrarily decided to use 4F11 antibody to coat ELISA plate wells and biotin-labeled 1C6 antibody to detect DAF captured on the 4F11-coated ELISA plates. With this combination of anti-DAF monoclonal antibodies, the new ELISA was sensitive to 0.8 ng/ml and accurate to 12.5 ng/ml (Fig. 2). DAF concentrations determined by the new ELISA correlated well, and in a

Table 1   Effects of centrifugation in the preparation of stool samples

	No.*	Stool DAF (ng/ml)	
		Centrifugation (+)	Centrifugation (−)
Control	1	0	0
	2	41.5	43.4
	3	35.8	145.2
	4	0	0
Colorectal cancer	1	73.2	71.6
	2	292.0	284.4
	3	12.0	24.4
	4	106.1	123.8
	5	0	6.9
	6	17.1	35.2

Stool samples were centrifuged either at 20,000 g for 15 min (Centrifugation (+)) or in a low-speed benchtop microcentrifuge for 30 sec (Centrifugation (−)). Supernatants were collected and measured for DAF concentration by the original ELISA using a combination of mouse monoclonal and rabbit polyclonal anti-DAF antibodies. *, sample number.

Table 2   Effects of detergents in the preparation of stool samples

	No.	Sample buffer	Detergents	
			Sodium deoxycholate	Octyl β-glucoside
Control	5	1.8	7.1	5.9
	6	3.3	9.0	8.6
	7	3.1	3.0	4.1
	8	0	0	0.1
Colorectal cancer	7	4.5	6.2	6.9
	8	79.1	76.5	84.7
	9	49.3	69.5	44.9
	10	138.7	125.6	129.2

Stools were suspended in sample buffer containing either octyl β-glucoside (60 mM) or sodium deoxycholate (9 mM) incubated at 4 °C for 30 min and centrifuged in a low-speed benchtop microcentrifuge for 30 sec. Supernatants were collected, and the amount of DAF was measured (ng/ml) by the original ELISA. Sample buffer: PBS containing 1% BSA, 0.05% Tween 20, and 1 mM phenylmethylsulfonylfluoride.

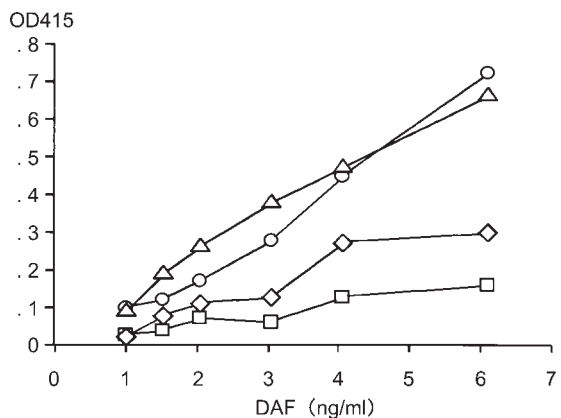linear manner (r = 0.999, $P < 0.0001$), with the concentrations obtained using the previous ELISA (Fig. 3).

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Fig. 1   ELISA using only monoclonal anti-DAF antibodies. ELISA plates were coated with 4F11, 5B2, or 1C6 anti-DAF monoclonal antibody. Plates were then incubated with various amounts of purified DAF. After washing, either biotin-labeled 1C6 antibody or biotin-labeled 4F11 antibody was added, followed by HRP-labeled streptavidin and ABTS as the substrate. ○, 4F11 + biotin-labeled 1C6; △, 1C6 + biotin-labeled 4F11; ◇, 5B2 + biotin-labeled 4F11; □, 5B2 + biotin-labeled 1C6.
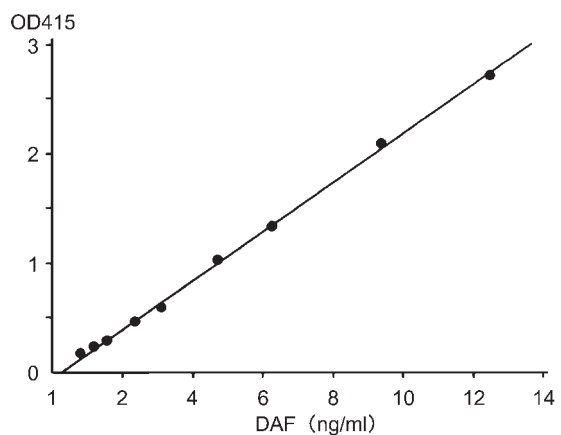
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Fig. 2   The dynamic range of the new ELISA system. ELISA plates were coated with 4F11 anti-DAF antibody and incubated with various amounts of purified DAF. After washing, biotin-labeled 1C6 antibody, HRP-labeled streptavidin and ABTS were sequentially added to the plates.
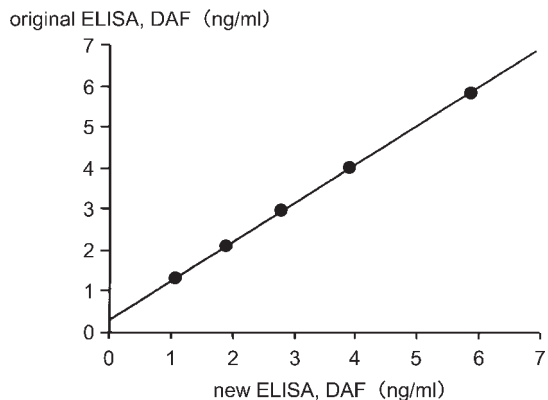
original ELISA, DAF (ng/ml)

Fig. 3   The relationship between DAF concentrations as determined by the new and the original ELISA. Various amounts of purified DAF were measured by the new ELISA using 4F11 and biotin-labeled 1C6 anti-DAF antibodies and by the original ELISA using the polyclonal and monoclonal anti-DAF antibodies. The correlation between the 2 assays was r = 0.999 ($P < 0.0001$).

Discussion

In this study, we refined the existing ELISA system for measurement of stool DAF for the detection of colorectal cancer. We investigated the effects of high-speed centrifugation of stool suspensions and also considered the effects of adding detergents to sample buffers for the suspension of stool samples. We found that high-speed centrifugation, a cumbersome process requiring an expensive high-speed centrifuge, could be omitted; the removal of large undigested food residues via short-duration centrifugation in a low-speed benchtop microcentrifuge sufficed to prepare the stool samples. The present results will enable the routine use of stool DAF testing, even at small clinic laboratories. In our previous study on DAF in the culture supernatants of HT-29 human colon cancer cells [10], nearly half of the DAF was precipitated by centrifugation at 100,000 g, and about one-fourth of the DAF was precipitated by centrifugation at 15,000 g. The previous findings suggested that a significant amount of DAF released from cultured HT-29 human colon cancer cells was associated with large fragments such as detached whole cells and cell debris, which were precipitated by centrifugation at 15,000 g. Thus, we expected to find an increase in the amount of DAF present in stool specimens by omitting high-speed centrifugation; however, the amounts of stool DAF did not change remarkably with a change in sample preparation. Nonetheless, we found that the cumbersome process of high-speed centrifugation could be avoided by using our refined assay system.

In addition, we examined the effects of 2 detergents, octyl β-glucoside and sodium deoxycholate, on sample preparation. DAF protein is anchored to the outer leaflet of the plasma membrane by a covalently attached glycosyl-phosphatidylinositol (GPI)-anchor [15]. As stated above, our previous study demonstrated that nearly half of the DAF in culture supernatants of HT-29 cells was present in the membrane-bound form, which was precipitated by centrifugation at 100,000 g [10]. GPI-anchored proteins are known to be resistant to solubilization by detergents such as Triton-X [16–19] but that they are soluble in octyl β-glucoside and sodium deoxycholate [18, 19]. Accordingly, addition of detergents such as octyl β-glucoside would be expected to solubilize membrane-bound DAF and thereby influence DAF measurement. However, in this regard, we found no significant difference. Thus, we decided to use the original buffer

(PBS containing 1% BSA, 0.05% Tween 20, and 1 mM phenylmethylsulfonylfluoride) for the sample preparations.

Among the 3 monoclonal anti-DAF antibodies studied in this study, combination with the 5B2 antibody yielded low OD values. We thus decided to use 4F11 and 1C6 antibodies in the new ELISA. The observed differences between OD values can be attributed to either the affinity of the antibody to DAF or to the epitopes of these antibodies. Fortunately, with the use of 4F11 and 1C6 monoclonal antibodies, we could refine the ELISA by using only monoclonal antibodies. Results obtained with the refined system correlated well with those obtained using the previous ELISA. This alteration of the previous method allowed us to achieve a stable ELISA for the measurement of stool DAF using a uniform source of antibodies; the results should enable routine use of the DAF assay for the detection of colorectal cancer.

DAF is known to be resistant to proteolytic enzymes [20] and is stable during heat treatment [21]; we previously demonstrated that measurement of stool DAF yielded consistent results during 24 h after defecation, even when samples were kept at room temperature [22]. These properties allow patients to send stool samples by mail, and this convenience facilitates compliance with colorectal cancer screening. One disadvantage of the current ELISA system is the dynamic range of the assay. The assay was accurate to 12.5 ng/ml, but the amount of DAF in stool specimens was often above that level. In such cases, we had to dilute stool specimens appropriately to fit the DAF concentration within the assay range. The development of an assay system with a wider range would overcome this inconvenience and is the next important step required to implement the viable clinical use of stool DAF measurement.

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