Characterization of Serum and Urinary Chromogranin A by Size Exclusion Chromatography: Impact on Calibrator Selection and Urinary Assay

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Serum chromogranin A (CgA) is a useful marker for neuroendocrine tumors and is detectable in carcinomas at advanced stages. Elevated serum CgA is also an indicator of poor prognosis in prostate cancer and is useful for predicting the failure of hormonal therapy for prostate cancer patients. We found that CgA molecules with three different sizes could be detected in normal human serum. However, only the largest CgA molecule appears in patients with liver disease. Serum taken from cancer patients is composed predominantly of the middle-sized molecule, whereas the smallest CgA molecule was elevated in serum drawn from renal patients. Moreover, only the smallest CgA

molecule was found in urine. We believe that the largest CgA molecule is metabolized by the liver, whereas the smallest CgA molecule is removed from the blood circulation via the kidney. Because the medium-sized CgA is the dominant molecule in both the cell medium of the tumor cell line SK-N-AS and sera from patients with malignant diseases, CgA from the cell medium was selected as the calibrator for the CgA ELISA assay. Our findings also suggest that it would not be possible to measure the urinary CgA to reflect the serum CgA concentration in order to detect pheochromocytoma among patients with hypertension. *J. Clin. Lab. Anal.* 15:193–198, 2001. © 2001 Wiley-Liss, Inc.

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Serum chromogranin A (CgA) is a well-known marker for neuroendocrine cells. It is useful for the diagnosis and management of patients with carcinoid-like tumors and neuroendocrine tumors such as neuroblastoma, pheochromocytoma, and small-cell lung carcinoma (1,2). CgA was found to be more stable and thus more easily manageable (3–6) than plasma and urinary catecholamines, which are traditionally used for the diagnosis and management of pheochromocytoma. Eisenhofer et al. (7) pointed out that the detection of pheochromocytomas in patients at risk for these tumors, such as patients with von Hippel-Lindau disease or multiple endocrine neoplasia type 2 (MEN-2), is hindered by the inadequate sensitivity of commonly available biochemical tests. Serum CgA was also found to have a sensitivity of 91% and specificity of 100% for the diagnosis of neuroblastoma (3–6); diagnosis of neuroblastoma usually relies on the determination of urinary vanillylmandelic acid (VMA) and homovanillic acid (HVA).

CgA detected in the tumor tissues of carcinomas has been shown to be associated with poor prognosis on the basis of immunohistochemical techniques (1,8). Recently we found that elevated levels of serum CgA could also be detected in patients with carcinoma of the prostate, breast, ovary, pan-

creas, and colon (9). Because serum CgA levels in all carcinomas appeared to parallel changes of serum-dominant tumor markers and were associated with sera containing highly elevated tumor markers, elevated serum CgA can also indicate a poor prognosis. We also found that in almost one-third of all prostate cancer patients undergoing hormonal treatment, serum CgA not only was elevated, but the elevation was also detected at an earlier stage when patients developed hormonal resistance, allowing early changes of a more effective therapy to be created (10).

In view of its clinical utilities, a CgA assay was developed in-house because commercial CgA assay is not available in the U.S. (11). However, we learned recently that circulating CgA is heterogeneous in size (12). At least three circulating CgA, in different hydrodynamic sizes of 600 kilodalton (kDa), 100 kDa, and 55 kDa, can be found in human serum. In order to accurately match the assay calibrator with the sample analyte for accurate assay results, a calibrator with the same

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TABLE 1. Outline of assay parameters for microplate CgA ELISA^a

Major step	Material	Method
Coating Ab	DAKO A430 pAb	150 μ L, 5 μ g/mL, 4°C, overnight
Sample + detecting Ab	100 μ L sample + 50 μ L 1% PBS-BSA + 10 μ L DAKO M0869 mAb	1:200 dilution, overnight, rt
Signal Ab	Amdex:HRP-anti-mouse Ab	1:5,000 dilution, 1 hr, rt
Substrate	150 μ L TMB	A450 nm, 15 min

Abbreviations: rt, room temperature; Ab, antibody. Coating buffer: 0.1 M carbonate buffer, pH 9.6. Blocking solution: PBS containing 1% BSA, 0.05% Tween 20, 0.05% casein, and 2% sucrose. Washing solution: PBS containing 0.05% Tween 20. Calibrator: cell culture medium of SK-N-AS cells without further purification.

size as that of the major CgA molecule that appeared in the serum sample was selected. We had two choices for the calibrator. We could pool serum from patients with renal diseases or use CgA from the cell medium of a SK-N-AS cell culture.

It is well known that pheochromocytoma can be detected among patients with hypertension. The detection of pheochromocytoma has relied on the measurement of urinary catecholamines, a procedure that has always been associated with certain technical difficulties (13). We developed a more sensitive assay to measure urinary CgA in an attempt to replace the reliance on the measurement of urinary catecholamines. However, we could not find an acceptable correlation between the levels of urinary CgA and serum CgA. The present study was designed to provide us with an explanation.

MATERIALS AND METHODS

The prepacked column containing Superose 12 H R (10/30, 24 mL) and FPLC, an automated HPLC system, were both from Pharmacia Biotech (Piscataway, NJ). Polyclonal rabbit anti-human CgA antibodies and monoclonal mouse anti-human CgA antibody, horseradish peroxidase (HRP)-conjugated monoclonal mouse anti-human CgA antibodies were obtained from Dako (Carpinteria, CA). The immunogen is the C-terminal 20-kDa fragment of CgA molecule). SK-N-AS neuroblastoma cell line was purchased from American Tissue Culture Collection (ATCC, Rockville, MD).

Microplate ELISA for CgA

An in-house developed ELISA on a microplate was used for measuring CgA in both serum and eluates from Superose 12 column chromatography. Details of the procedure have been described in our earlier reports (10,11). The assay format is outlined in Table 1. Both pooled serum from renal patients with urine creatinine greater than 6 mg/dL and a cell medium of SKBr3 cell culture have been used as calibrators and were calibrated against CIS RIA.

Superose 12-Column Chromatography

Normal serum and sera from patients with cancers and renal disease were subjected to size exclusion chromatography

on columns containing Superose 12 mounted on FPLC. The buffer used for column equilibration and elution was 0.05 mol/L phosphate buffer containing 0.15 mol/L, pH 7.2. The flow rate was maintained at 0.5 mL/min during chromatography. Eluate was collected in tubes at 0.5 mL/tube for the measurement of CgA by an in-house ELISA. Usually 100 μ L of specimen, serum or urine, containing at least 500 ng/mL of CgA were injected for HPLC determination.

Fig. 1. CgA immunoreactive elution profile of Superose 12 chromatography of normal adult serum. Three CgA immunoreactive peaks were identified in the elution profile: L, the largest CgA molecule; M, the medium-sized molecule; and S, the smallest CgA molecule. (A) 100- μ L aliquot of a normal serum without any abnormal tumor markers and chemistry profile containing 98 ng of CgA. (B) 100- μ L aliquot of cord serum from a newborn baby containing 151 ng of CgA. V_0 , void volume; V_t , total volume.

Superose 12: malignant diseases

Fig. 2. CgA immunoreactive elution profile of Superose 12 chromatography of sera from patients with various malignant diseases. It appears that the majority of the CgA molecules found in the serum of all malignant diseases examined is made up of the medium-sized molecule. However, in addition to the medium-sized CgA, the largest (L) CgA molecule was also found in serum from patients with hepatoma. (A) 100 μ l of SK-N-AS cell medium

diluted with BSA-PBS (0.1 mol/l phosphate-buffered saline at pH 7.2 containing 1% bovine serum albumin). (B) 100 μ l of serum from a patient with ovarian disease. (C) 100 μ l of serum from a patient with primary hepatoma. (D) 100 μ l of serum from a patient with lung cancer. (E) 100 μ l of serum from a patient with pheochromocytoma. (F) 100 μ l of serum from a patient with neuroblastoma.

RESULTS

In Normal Serum

Despite the relatively low concentrations of CgA contained in normal serum, we could detect three CgA molecules of different size in the normal serum (Fig. 1). The peak corresponding to the third smallest CgA molecule most likely emerged with the CgA molecule peak of medium size and appeared only as a broad shoulder. The largest CgA molecule emerged behind the void volume of the column and eluted at about 13–14 min. The second CgA molecule of medium size appeared immediately before albumin, or at an elution time of 20 min. The last peak, corresponding with the smallest of these three CgA molecules, appeared behind the position of albumin and eluted from the column at about 24 min. The presence of the smallest CgA molecule in the serum is supported by the elution profile of cord serum in Figure 1B. The relatively high third peak could be due to the immaturity of the kidney of a newborn baby.

In Serum From Patients With Malignant Diseases

It is rather surprising that the medium-sized CgA molecule was found to be prevalent in all sera from patients with various malignant diseases (Fig. 2). The same molecule was also detected as the major CgA molecule in the cell medium of the neuroblastoma tumor cell culture (Fig. 2A). Because the calibrator should be the same molecule as the analyte of interest, CgA from the SK-N-AS cell culture was used as the calibrator for our CgA immunoassay. The elution profile of CgA activity from the serum of patients with hepatoma (Fig. 2C) was not typical of all other elution profiles derived from patients with malignant diseases. In addition to the medium-sized CgA molecule, there were also significant amounts of the largest molecule appearing in the elution profile, similar to what we found in the elution profile from patients with liver diseases (Fig. 3C). We suspect that patients with hepatoma may have impaired liver function, causing elevation of the largest CgA molecule in the serum.

In Serum and Urine From Patients With Renal Disease

We found that the serum of patients with renal insufficiency contains elevated serum CgA and that the majority of the elevated serum CgA was due to the elevation of the small CgA molecule (Fig. 3A). It is interesting to note that only the smallest CgA molecule was detectable in urine (Fig. 3B).

Superose 12 Chromatography

Fig. 3 CgA immunoreactive elution profiles of Superose 12 chromatography. It appears that the smallest (S) CgA molecule in the urine is secreted into the urine. Patients with renal diseases accumulate S-CgA in the serum due to their inability to clear the small CgA through kidney. (A) 100 μ l of pooled serum from patients with renal insufficiency. (B) 100 μ l of urine containing more than 500 ng/ml of CgA. (C) 100 μ l of serum from a patients with liver disease.

In Serum From Patient With Liver Disease

Elevated serum CgA is often found in patients with liver diseases (Fig. 3C). There was a slight correlation between the level of serum CgA and abnormal liver enzyme activities. The elution profile of CgA activity suggested that the elevated activity was derived from the elevated level of the largest CgA molecule, shown in Fig. 3C. It appears that the elevation of serum CgA in liver patients was due to the inability of liver patients to metabolize the large size of the CgA molecule.

Slope Comparison

The slope of a calibration curve reflects the affinity of the calibrator for the antibody used in the assay. An ideal calibration curve should have a slope identical to that of the sample analyte; otherwise, dilution of a specimen will produce values different from that of the undiluted sample after correcting for the dilution factor. As shown in Figure 4, the slope of CgA from the cell medium superimposes exactly on that of the medium-sized CgA molecule. On the other hand, the slopes of both the smallest and the largest CgA molecules reveal slight deviations from the slope of cell medium CgA. Thus, the cell medium CgA was selected as the calibrator for our CgA assay.

DISCUSSION

Our results confirm the report by Corti et al. (12) that CgA molecules of different sizes are present in the serum. The results are interesting but not surprising, as CgA has been known to be subject to proteolysis, and its fragments have different physiological activities (14). The intact CgA is a 68 kDa, acidic protein (isoelectric point, pI, 4.57–4.68) (1,8). Both the medium- (M) and small-sized CgA molecules are eluted immediately before and after the albumin peak, respectively, which suggests that their molecular weights are close to 68 kDa. The medium-sized molecule is most likely the intact CgA molecule, and the smallest CgA molecule could be the degradation product of the M molecule, because the cell medium CgA molecule is not likely to have been subjected to proteolysis. The largest CgA molecule appearing close to the void volume could then be the polymer or aggregate of intact CgA. It is apparent that further investigation will be required to confirm this.

The unusual CgA immunoreactive elution profiles in serum from patients with liver and renal disease are also of interest. The appearance of the large CgA molecule in hepatoma patients and the small CgA molecule in urine suggests that the large CgA molecule is metabolized by the liver and that the small molecule is cleared via the kidney. The relatively high concentration of small CgA molecule in newborn baby serum further supports the fact that the small CgA molecule is normally secreted into the urine. The kidney of a

Fig. 4. Comparison of slopes of isolated CgA molecule of three different sizes (L, M, and S CgA) with that of cell medium CgA. The complete correspondence of slope between cell medium CgA and M-CgA from cancer patients suggests that CgA from the cell medium should be selected as assay

calibrator. The CgA value assigned to serum CgA molecules was based on dilution. Only the highest value of individual CgA molecule was determined by the assay. The CgA value of cell medium CgA was calibrated against the CIS radioimmunoassay CgA kit (CIS Bio International, France).

newborn baby may not have matured enough to perform normally, thus resulting in the accumulation of the small CgA molecule in the blood circulation. The elevation of serum CgA in patients with liver disease and liver cancer and in patients with renal insufficiency is most likely due to the inability of these patients to remove the CgA from blood circulation.

It is well known that for an immunoassay to provide accurate assay values it is important to use a calibrator that has the same affinity for the assay antibody as the sample analyte. To prepare the calibrator for the CgA assay we have attempted to collect CgA from the medium of an SK-N-AS cell culture, a neuroblastoma tumor cell line. Interestingly, the medium of

Fig. 5. Correlation of concentration of urinary CgA with that of serum CgA of same individuals. A poor correlation with correlation coefficient (0.355) was found between urinary and serum CgA of the same individuals.

the cell culture contained only the medium-sized CgA molecule, similar to what was found in cancer patients. We have also pooled serum from patients with renal diseases containing elevated serum concentration of CgA with urinary creatinine greater than 6 mg/dl (8). The majority of CgA molecules found in the pooled serum contained predominantly the small CgA molecular form. The use of pooled serum as calibrator has the advantage of having the same matrix as the sample and is relatively inexpensive as well. However, because the application of the CgA assay is reserved mainly for patients with malignant diseases, CgA from the cell medium became the choice as ideal calibrator.

The measurement of urinary catecholamines has been utilized to identify pheochromocytoma among patients with hypertension. Because CgA is an excellent marker for pheochromocytoma, we made an attempt to develop a sensitive CgA assay to measure CgA in the urine to replace the catecholamine measurement in the urine in order to better identify patients with pheochromocytoma. We were initially very puzzled by the lack of correlation between urinary CgA and CgA in the serum (Fig. 5). Later, we found that only one of the three CgA molecules present in the serum was secreted into the urine; the largest and medium-sized CgA molecules were not. In fact, the CgA concentration in the urine was much lower. A 5-fold more sensitive CgA assay is required to measure urinary CgA. It appears that in order to establish the clinical utility of the urinary CgA assay, we need to establish the reference interval for urinary CgA and focus only on CgA concentration in the urine and ignore the correlation with serum CgA. Using the urinary CgA, we need to differentiate patients with pheochromocytoma from patients with hypertension. The urinary CgA assay may also be useful to identify patients with carcinoid tumors with elevated urinary serotonin.

The selection of CgA from the cell medium of a SK-N-AS cell culture is also supported by the slopes exhibited by various CgA molecules. Slopes of the dose-response curve of CgA from the SK-N-AS cell medium and the predominant CgA from patients with malignant diseases are almost identical, which encourages the use of CgA from SK-N-AS as assay calibrator. The slopes from both small and large CgA molecules appear to be slightly deviated from that of medium-sized CgA, suggesting that they may not be suitable as calibrators, especially for assaying CgA in serum from cancer patients.

Measurements of plasma normetanephrine and metanephrine have been recently pointed out by Eisenhofer et al. (7) as being useful in screening for pheochromocytomas in patients with a familial predisposition to these tumors. It would be interesting to know whether plasma CgA is also useful in this regard.

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