Serum Prolidase Activity as a Biomarker for Choroid Plexus Calcification

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The choroid plexus (CP) performs multiple functions such as secretion and reabsorption. CP also acts as the blood-cerebrospinal fluid barrier. Prolidase plays an important role in collagen metabolism by degrading imidodipeptides, in which proline or hydroxyproline residue is located at the C-terminal end. Serum prolidase activity (SPA) may reflect the degree of fibrosis and inflammation. Choroid plexus calcification (CPC) is considered as the physiological calcification of the brain, and CPC is diagnosed by the presence of calcification in the anatomical region on computed tomography (CT). Here, CPC and non-calcified CP were defined by Hounsfield Units (HU) values of > 150 and < 50, respectively. We aimed to measure SPA in subjects with CPC and those with non-calcified CP. This study included 89 subjects who were admitted to the neurology clinic and underwent CT: 44 subjects with CPC and 45 subjects with non-calcified CP. The neurological examination of all subjects was normal; namely, the subjects with CPC were asymptomatic. The SPA level was significantly higher in the CPC group than that in the non-calcified CP group ($p < 0.002$), and there was a significant positive correlation between vitamin D and SPA levels in the CPC group. In contrast, the vitamin D and parathyroid hormone levels were higher in the CPC group, but the difference was not statically significant ($p > 0.05$). These findings indicate that SPA is a biomarker for CPC that may be predictive of future brain disease.

Introduction

The choroid plexus (CP) is located in the superior part of the inferior horn of the lateral ventricles of the brain and performs multiple functions such as secretion and reabsorption. CP acts as a blood-cerebrospinal fluid (CSF) barrier. Through this barrier, essential molecules required for brain homeostasis pass into CSF. Epithelial cells of CP play an effective role in barrier function and synthesize prostaglandins and growth factors (Serot et al. 2001).

CP calcification (CPC) is associated with age and sex (Kwak et al. 1988). Electron microscopy of CP reveals precipitates of calcium (Ca) in the subepithelial regions and in the walls of blood vessels, but they are mainly observed in spherical psammoma bodies (Alcolado et al. 1986). The most important factors associated with the occurrence of tissue calcification are as follows: extracellular matrix (ECM) suitable for mineralization, extracellular levels of inorganic phosphate (P) and Ca, and levels of mineraliza-tion inhibitors that may be systemically or locally expressed (Brylka and Jahnen-Dechent 2013; Ceylan et al. 2015). CP degeneration may result in low CSF production and hyperthermic brain injury in animals. CP is highly vulnerable to damage on head injuries, infections, and ischemic conditions (Maxwell et al. 1992). Furthermore, Palha et al. (2012) mentioned acute peripheral inflammation in CP.

Collagen, the most abundant protein of ECM in mammalian tissues, maintains the architecture and integrity of connective tissue. Proteases catalyze the degradation of proteins into smaller peptides and amino acids. Prolidase (EC3.4.13.9) is a unique cytosolic protease that degrades imidodipeptides, in which a proline or hydroxyproline residue is located at the C-terminal end (Miltyk et al. 2007). Prolidase plays an important role in the recycling of proline from imidodipeptides for the resynthesis of collagen, whereas hydroxyproline is not a substrate for recycling and is excreted into urine (Miltyk et al. 2007). Prolidase activity (PA) is detected in plasma and in various organs, such as

the liver, brain, heart, uterus, and thymus (Kaleli et al. 2006; Kitchener and Grunden 2012; Arikanoglu et al. 2013). Prolidase requires manganese-II and reducing conditions for optimal activity. In humans, cytosolic prolidase catalyzes the final step of protein catabolism where it serves to liberate proline from proline-rich dietary and endogenous proteins such as extracellular collagen. Fluctuations in PA indicate dysfunctional collagen metabolism, which is characteristic of many diseases, as well as disease progression (Miltyk et al. 2007; Besio et al. 2010; Kitchener and Grunden 2012).

The objective of the present study was to investigate serum PA (SPA) and calcification markers including Ca, parathyroid hormone (PTH), and vitamin D in subjects with or without CPC to assess the relationship between PA and CPC.

Materials and Methods

The study was conducted at the Medicine Faculty, Sakarya University, Sakarya, Turkey. The subjects were introduced by the Department of Clinical Neurology. The ethics committee of Sakarya University, Medicine Faculty, approved this study, and all subjects provided informed consent.

Subjects

The groups comprised subjects without brain pathology determined using computed tomography (CT). The inclusion criteria were as follows: 1) asymptomatic subjects, 2) normal medical examination results, and 3) subjects with or without CPC on CT. We excluded subjects with acute and chronic inflammatory disease, diabetes mellitus, renal failure, acute-chronic liver failure, and malignancy. This study included 89 subjects: 44 with CPC (CPC group) and 45 with non-calcified CP (control group). The CPC group comprised 20 males and 24 females between 17 and 45 years of age (range, 29.70 ± 10.23 years), while the control group comprised 26 males and 19 females between 16 and 45 years of age (range, 32.22 ± 8.04 years).

Samples

Blood samples collected from subjects who fasted overnight were collected into tubes without EDTA, centrifuged at 4°C at 3,000 rpm for 10 min. The serum was immediately separated from the cells after centrifugation. Serum samples for measuring PA were stored at −80°C, and the levels of vitamin D, Ca, and PTH were measured. All measurements were taken using the same series after the samples were thawed.

Prolidase assay

The method of SPA measurement was defined by Myara et al. (1982), and we used the method optimized by Ozcan et al. (2007). PA was measured using a spectrophotometric method that detects proline produced by prolidase. Reaction conditions were as follows: 100 μl of serum and 500 μl of preincubation solution (50 mmol/l Tris-HCl buffer pH7.8, 1 mmol/l reduced glutathione, 5 mmol/l MnCl$_2$, and 0.1% Triton-X100) were mixed, incubated at 37°C for 3 h, and 100 μl was added to 100 μl of Gly-Pro (144 mmol/l). This solution was incubated at 37°C for 30 min. Trichloroacetic acid (1 ml, 0.45 mol/l) was added to terminate the reactions. This mixture was centrifuged at 1,500 rpm for 5 min, and 500 μl of supernatant was used to measure the proline concentration using a modification of Chinard's method (Chinard 1952; Myara et al. 1982).

Assays for vitamin D, Ca, and PTH

The vitamin D, Ca and PTH levels of the samples were measured using commercial kits. Chemiluminescence assays were used to detect vitamin D and PTH. Vitamin D levels were determined using an in vitro diagnostic kit (*IDS*-iSYS 25-Hydroxy Vitamin D[S], lot number: 2215, Immunodiagnostic Systems Limited, Boldon, UK) with an IDS-iSYS Multi-Discipline Automated System. The PTH assay was conducted using an in vitro diagnostic kit (Architect Intact PTH kit manufactured for Abbott Laboratories, lot number: 02213K000; Biokit S.A., Barcelona, Spain). Colorimetric assays of Ca were performed using an Architect Automated System (lot number: 87290UN14; Abbott Laboratories, Abbott Park, IL, USA).

Imaging analysis

All CT examinations were performed using a 4-row multidetector CT scanner (Somatom Spirit, Siemens Healthcare, Forcheim, Germany). CP density was measured using the region of interest. The mean densities described using Hounsfield Units (HU) of > 150 HU or < 50 HU were defined as CPC or non-calcified CP, respectively. One radiologist performed the measurements, and the calculations were performed for each subject.

Statistical analysis

The Kolmogorov-Smirnov test was used to evaluate whether the values of the variables were normally distributed. A t test of independent sample groups was used to compare parametric continuous data. The Mann-Whitney test was used to compare nonparametric continuous data. Continuous data are presented as the mean ± standard deviation. Spearman's or Pearson's correlation coefficient was used to determine the statistical significance of the differences between variables. Categorical variables were compared using the chi-square test and they represented as counts and percentages. A p value of < 0.05 was considered statistically significant. Analyses were performed using SPSS Statistics, Version 22.0 (IBM Corp, Armonk, NY, USA).

Results

CT images of subjects with CPC and those with non-calcified CP (control group) are shown in Fig. 1. Here we used CT to determine the HU values of the CPC and control groups. The CPC and control groups were defined by HU values of > 150 and < 50, respectively. HU values are shown in Table 1. Other features are also summarized in Table 1, including the levels of SPA, Ca, PTH, and vitamin D, as well as the age and sex. The distribution of age and sex between the groups was not significantly different. When compared with the control group, vitamin D and PTH levels of the CPC group were higher, although the differences were not statistically significant (p > 0.05). Ca levels were similar in both groups (p > 0.05). When compared with the control group, SPA and HU values of the CPC group were significantly higher (p < 0.002, p < 0.001, respectively).

The correlations between the parameters of the subjects with CPC are shown in Table 2. There was a signifi-
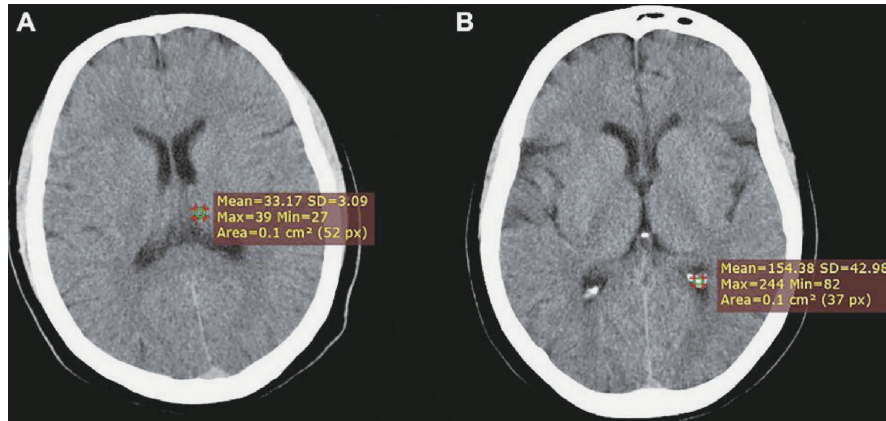
Fig. 1. CT imaging of the choroid plexus.
Shown are the axial CT images of non-calcified (A) and calcified (B) choroid plexus with given density measurements by means of Hounsfield Units (HU).

Table 1. SPA, Ca, PTH, and vitamin D levels in the CPC and control groups.

	CPC group (n = 45)[1]	Control group (n = 45)	p
Sex (males)	15 (33.3)	23 (51.1)	0.135
Age (years)	28.71 ± 9.87	32.22 ± 7.74	0.064
Ca (mg/dl)	9.19 ± 0.61 (n = 39)	9.35 ± 0.51 (n = 36)	0.208
PTH (pg/ml)	83.41 ± 90.1 (n = 39)	68.56 ± 37.18 (n = 35)	0.588*
Vitamin D (ng/ml)	10.03 ± 13.3 (n = 39)	7.92 ± 5.16 (n = 34)	0.328*
HU values	166.44 ± 134.45	35.09 ± 7.99	**0.001**
SPA (U/l)	1,273.78 ± 261.88 (n = 44)	1,118 ± 181.95	**0.002**

Data represent the mean ± standard deviation and the number of subjects (n).
*Mann-Whitney test.
[1]Each group includes males and females.
The p values indicating statistical significance are boldfaced.

Table 2. Correlation of SPA, Ca, PTH, and vitamin D levels, as well as HU values, in subjects with CPC.

Parameters	Ca	PTH	Vitamin D	HU values
PTH	−0.222			
Vitamin D	−0.272	−0.255		
HU values	0.124	−0.272	0.068	
SPA	−0.249	0.166	**0.425***	−0.231

*Significant positive correlation.

cant positive correlation between vitamin D and SPA levels. There was no significant correlation between other parameters.

Discussion

The analyses of the biochemical markers of calcifica-tion indicate the validity of the classification used in this study. We showed that SPA levels were significantly higher in the CPC group. In contrast, there were no significant differences in the levels of Ca, vitamin D, and PTH between the two groups.

Prolidase is an iminodipeptidase that catalyzes the

rapid hydrolysis of the peptide bond formed between the imino nitrogen of proline or hydroxyproline (Myara et al. 1984a, b; Yaron and Naider 1993). Increased PA indicates fibrosis. Therefore, in patients with fibrosis, this enzyme contributes to an increase in collagen levels (Yaron and Naider 1993; Abraham et al. 2000). A previous study reported a correlation between inflammation and PA, such as in the pathogenesis of lung fibrosis (Yaron and Naider 1993). There have been several studies that correlate inflammation with PA. PA and inflammation can be correlated to each other during the process of lung fibrosis (Turkbeyler et al. 2012). PA is associated with different diseases such as Alzheimer disease, diabetic neuropathy, bipolar disorder, lung diseases, and non-ulcer dyspepsia (Erbagci et al. 2002; Selek et al. 2011; Uzar et al. 2012; Arikanoglu et al. 2013; Kumari et al. 2013). In our study, we found that the mean SPA level in the CPC group was higher than that in the control group, although these results are not characteristic of patients with inflammatory diseases that affect the liver and kidneys or other organs.

A literature search did not identify any studies pertaining to SPA in subjects with CPC, although one study on patients with subarachnoid hemorrhage found that the concentrations of the C-terminal propeptide of type I procollagen and the N-terminal propeptide of type III procollagen were higher in CSF than in serum (Sajanti and Majamaa 1999). Our present findings led us to conclude that SPA increases if the synthesis of collagen isoforms increases in CP.

The level of fetuin-A, which is a marker of inflammation, is significantly higher in patients with CPC (Ceylan et al. 2015). Similarly, we show here that SPA in subjects with CPC was significantly increased compared with that in controls, suggesting that CPC developed because of inflammation. Further, we hypothesize that calcification of CP causes fibrosis.

An increase in the plasma concentration of Ca leads to the calcification of soft tissues (Bender 2009), and intracranial physiological calcification frequently occurs (approximately 35.2%) in adults (Sedghizadeh et al. 2012). Further, majority of physiological calcification occurs in a region occupying 12%-66.2% of CP (Daghighi et al. 2007; Sedghizadeh et al. 2012). We show here that CPC (which is considered as the physiological calcification) was independent of the calcification markers including Ca, vitamin D, and PTH. Further, significant increases in PA in the CPC group may play a role in cerebral inflammation.

Evidence indicates that CPC is associated with degenerative changes that accompany aging (Kieffer and Gold 1974) and that the severity of CPC progressively increases with age (Makariou and Patsalides 2009). Because of the small number of subjects studied here, we were unable to correlate our findings with age and sex. Therefore, the aim of our future studies is to assess the significance of the association between CPC and elevated PA.

The small degree of CPC of the adult lateral ventricles mainly develops symmetrically and does not usually cause symptoms, and extensive calcification of CP is very rare. However, physiological and seemingly innocuous calcification may cause cerebral neuroinflammation (Picht et al. 2004). The neuroinflammation raises an alarm for the presence of neurodegenerative processes. Further, calcification may influence neurodegenerative processes that occur after cerebral neuroinflammation. Therefore, controlled studies that aim to determine the contribution of neuroinflammation are required to improve the treatment of neurodegenerative diseases.

In conclusion, SPA serves as a useful biomarker for physiological calcification that is detected early during the diagnosis of brain disorders. Moreover, the maintenance of SPA at low levels may contribute to the prophylactic treatment of neurodegeneration.

Author Contributions

Conflict of Interest

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