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Serum pyroglutamyl aminopeptidase activity: a promising novel biomarker candidate for liver cirrhosis

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Objective. As a reflect of tissue damage, serum aminopeptidases have been proposed as biomarkers of various diseases. In order to search new serologic markers for liver cirrhosis we conducted a preliminary study in which we analyzed a broad range of aminopeptidase activities in serum of controls and patients diagnosed with pancreatitis, hepatitis, and liver cirrhosis without distinction among the etiological type or the degree of severity of each condition.

Methods. Alanyl-, arginyl-, glutamyl-, cystinyl- pyroglutamyl-, and aspartyl-aminopeptidase activities were analyzed fluorometrically, using aminoacyl-β-naphthylamides as substrates. In addition, various parameters, such as alanine transaminase, aspartate transaminase, alkaline phosphatase, total bilirubin, direct bilirubin, and gamma glutamyl transpeptidase were assayed as routine laboratory test for liver function.

Results. Compared with control group, alanyl- and arginyl-aminopeptidase activities increased nonspecifically in pancreatitis, hepatitis and liver cirrhosis, glutamyl- and cystinyl-aminopeptidases did not differ between groups and pyroglutamyl-aminopeptidase demonstrated that while pancreatitis and hepatitis did not differ between them and with controls, this activity decreased selectively in liver cirrhosis compared with all the rest of groups ($p<0.001$ vs. control and $p<0.01$ vs. pancreatitis and hepatitis). Aspartyl-aminopeptidase also decreased significantly ($p<0.05$) in liver cirrhosis compared with controls. Routine parameters for liver function test increased, as expected, in the three pathologies analyzed.

Conclusions. Despite the heterogeneous composition of the three patient groups, the specific reduction of the levels of pyroglutamyl-aminopeptidase activity in serum of liver cirrhosis patients might be considered as a potential candidate to be included in a combination of markers for the diagnosis of this disease.

Key words: aminopeptidases, arylamides, serum biomarkers, liver cirrhosis

Despite the efforts in the search of serologic markers for liver cirrhosis (CIR), liver biopsy and subsequent histological examination is currently necessary for the diagnosis of the disease. Therefore, the search of non-invasive methods for the preferably early diagnosis of hepatic fibrosis is still necessary (Heidelbaugh and Bruderly 2006).

Serum aminopeptidases are zinc metalloenzymes used in clinical chemistry as markers of disease, usually because they are a reflection of tissue damage (Sanderink et al. 1988; Martinez et al. 1999), and there are simple, quick and reliable methods for their detection in serum and tissues (Villarejo et al. 2014). For example, alanyl-aminopeptidase is greatly increased in hepatobiliary

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diseases (Sanderink et al. 1988). However, the main restraint on the use of alanyl-aminopeptidase in differential diagnosis is that, as with other conventional serum tests, this enzyme is nonspecifically elevated in many digestive and non-digestive disorders (Sanderink et al. 1988). In addition, these enzymes also play a role in the regulation of several circulating biologically active peptides (Ramirez et al. 2008). Therefore, changes in these peptides may be reflected in enzymatic activity and vice versa.

In order to search new possible serologic markers for CIR we conducted a preliminary study in which we analyzed a broad range of aminopeptidase activities in serum of controls (Co) and patients diagnosed of pancreatitis (PA), hepatitis (HE) and CIR.

Subjects and Methods

Patients (aged 33 to 80 years) were diagnosed of PA (n=21, 10 males and 11 females), HE (n=21, 12 males and 9 females) and CIR (n=23, 11 males and 12 females) at the University Hospital San Cecilio (Granada, Spain). In the present preliminary study, no distinction about the etiological type or degree of severity of the diseases was made. The age-matched control group (n=26, 13 males and 13 females) was composed for healthy volunteers whose routine analytical determinations were within the range of normality. Blood samples were obtained by venipuncture between 8:00 and 9:00 a.m. after overnight fasting. Sera were obtained after coagulation of blood samples at room temperature and centrifugation for 15 min at 3000 x g. From each obtained serum, two aliquots were separated: one of them was used for routine analytical determinations in the same day of the extraction and the other frozen and stored at -80°C for the ulterior analysis of aminopeptidase activities. Hemolytic, icteric, or turbid samples were discarded. Alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), total bilirubin (TBIL), direct bilirubin (DBIL), and gamma glutamyl transpeptidase (GGT) were assayed as routine laboratory test for liver functional status by means of a standard automated technique. Before the study, patients and controls gave their informed consent and the Institutional Ethics Committee of the Hospital approved this research.

Alanyl- (ALAAP), arginyl- (ARGAP), glutamyl- (GLUAP), cystinyl- (CYSAP), pyroglutamyl- (PGLUAP) and aspartyl- (ASPAP) aminopeptidase activities were measured fluorometrically, using aminoacil-β-naphthylamides (aaNNap) as substrates in serum of patients and controls. All aminopeptidase activities were determined in triplicate, at the same day, in a fluorometric assay using the arylamides alanyl- (AlaNNap), arginyl- (ArgNNap), glutamyl- GluNNap), cystinyl- (CysNNap), pyroglutamyl- (pGluNNap) and aspartyl-β-naphthylamide (AspNNap) as the substrates as previously described (Greenberg 1962; Cheung and Cushman 1971; Schwabe and McDonald 1977; Tobe et al. 1980). Briefly, ALAAP, ARGAP and CYSAP were measured fluorometrically using AlaNNap, ArgNNap and CysNNap as substrates, according to the modified method of Greenberg (1962). 10 µl of each serum was incubated during 30 min at 25ºC with 1 ml of the substrate solution (2.14 mg/100 ml AlaNNap or 3.35 mg/100 ml ArgNNap or 5.63 mg/100 ml CysNNap), 10 mg/100 ml bovine serum albumin (BSA), and 10 mg/100 ml dithiothreitol (DTT) in 50 mM of phosphate buffer, pH 7.4, for AlaAP and ArgAP; and 50 mM HCl-Tris buffer, pH 6, for CysAP.

Pyroglutamyl-aminopeptidase was measured in a fluorogenic assay using pGluNNap as the substrate, according to the modified method of Schwabe and McDonald (1977): 25 µl of each serum was incubated during 240 min at 37ºC with 1 ml of substrate solution (2.54 mg/100 ml pGluNNap, 10 mg/100 ml BSA, 10 mg/100 ml DTT, 37.8 mg/100 ml EDTA in 50 mM of phosphate buffer, pH 7.4).

Aspartyl-aminopeptidase was determined fluorometrically with AspNNap as the substrate, according to the method of Cheung and Cushman (1971) modified as follows: 25 µl of each serum was incubated for 240 min at 37ºC with 1 ml of the substrate solution (2.58 mg/100 ml AspNNap, 10 mg/100 ml BSA, 10 mg/100 ml DTT and 39.4 mg/100 ml $MnCl_2$ in 50 mM HCl-Tris buffer, pH 7.4).

Glutamyl-aminopeptidase was also determined in a fluorometric assay using GluNNap as the substrate according to the method of Tobe et al. (1980) modified as follows: 25 µl of each serum was incubated during 240 min at 37ºC with 1 ml of the substrate solution (2.72 mg/100 ml GluNNap, 10 mg/100 ml BSA, 10 mg/100 ml DTT and 0.555 g/100 ml $CaCl_2$ in 50 mM HCl-Tris, pH 7.4).

All the reactions were stopped by adding 1 ml of 0.1 M acetate buffer (pH 4.2). The amount of β-naphthylamine released, as a result of enzymatic activity was measured fluorometrically at 412 nm emission wavelength with 345 nm excitation wavelength. Aminopeptidase activities were expressed as pmol of AlaNNap, ArgNNap and CysNNap, or nmol of AspNNap, pGluNNap and GluNNap hydrolyzed per minute per liter of serum.

For the statistical analysis, one-way analysis of variance was used to evaluate differences between groups. Post-hoc comparisons were made with Tukey's test, and p-values below 0.05 were considered significant.

Results

Results are presented in Fig. 1 and Table 1. No differences between male and female subjects were observed in the different groups studied. Compared with control group, ALAAP and ARGAP activities increased nonspecifically in PA, HE and CIR ($p<0.001$), GLUAP and CYSAP did not differ between groups and PGLUAP demonstrated that while PA and HE did not differ between them and with controls, this activity decreased significantly and selectively in CIR compared with all the rest of groups ($p<0.001$ vs. Co and $p<0.01$ vs. PA and HE). ASPAP also decreased significantly ($p<0.05$) in CIR compared with controls (Fig. 1). Routine parameters for liver function test increased, as expected, in the three pathologies analyzed (Table 1).

Discussion

Human serum aminopeptidases have been used, mainly in clinical biochemistry, as biomarkers for several diseases, particularly ALAAP (Sanderink et al. 1988), their levels increasing presumably via autolysis from cellular membranes or release of soluble enzymes following cellular damage (Reichling and Kaplan 1988; Ijima et al. 2002). Alternatively, it has been also proposed that aminopeptidases are secreted by endothelial cells to the blood stream. This secretion is in part modulated by autonomic activity (Villarejo et al. 2014).

Interestingly, the present results demonstrated a clear discrepancy: as expected, ALAAP and ARGAP increased nonspecifically in the different pathologies analyzed. In contrast, mainly PGLUAP but also ASPAP decreased specifically in CIR. This circumstance suggests in principle that the process of release of enzymes from hepatic cells could affect differentially to the diverse classes of aminopeptidases. On the other hand, since no distinction was made about the etiological type or degree of severity, it is remarkable that the highly significant decrease of PGLUAP in CIR was realized in spite of the heterogeneous characteristic of the three pathologies analyzed. It could be expected that the cell damage existing in CIR could account in part for the levels of serum PGLUAP, being these originated partially from the liver. However, the group of patients with CIR showed decreased, rather than increased PGLUAP in serum. In contrast with other enzymes, in hepatitis or pancreatitis PGLUAP was not significantly elevated. Therefore, cell damage and the subsequent release of enzymes was not the direct cause of altered serum PGLUAP in the case of CIR. Some particular and currently unidentified characteristics that this disease does not share with hepatitis or pancreatitis should be the probable cause of this significant decrease. Therefore, the specificity of this decrease in PGLUAP suggests that serum level of this enzyme may be useful in the diagnosis or prognosis of CIR.

Pyroglutamyl aminopeptidase is an omega peptidase, which removes the amino terminal pyroglutamate residue from specific pyroglutamyl substrates. According to Cummins and O'Connor (1998), the measurement of this enzyme in serum using pyroglutamyl-β-naphthylamide as substrate, correspond to the type I of the three forms of this enzyme identified in mammalian tissues. It is a cytosolic enzyme, widely distributed in adult tissues with high levels in kidney and liver (Fuse et al. 1990). Endogenous substrates for this type are

Table 1

Liver function test

Parameter	Control (n=22-26)	Pancreatitis (n=21)	Hepatitis (n=21)	Liver cirrhosis (n=23)	Reference limits [Units]
ALT	21.60±1.13	43.04±5.90*	314.9±132.4	107.04±19.10**	0-37 [U/l]
AST	19.72±2.19	65.09±11.50**	337.9±111.9	60.7±10.6**	0-40 [U/l]
ALP	143.0±7.7	366.7±46.1***	458.0±88.1*	295.0±24.3***	100-280 [U/l]
TBIL	0.67±0.06	1.36±0.35	2.59±1.11	4.05±1.09*	0-1 [mg/dl]
DBIL	0.236±0.020	0.537±0.160	1.11±0.53	1.75±0.48*	0-0.5 [mg/dl]
GGT	18.9±2.7	198.4±42.3**	84.4±24.0	106.8±19.6**	7-32 [U/l]

ALT - Alanine transaminase; AST - Aspartate transaminase; ALP - Alkaline phosphatase; TBIL - Total bilirubin; DBIL - Direct bilirubin; GGT - Gamma glutamyl transpeptidase. Values represent mean ± S.E.M. in corresponding units.
*p<0.05; **p<0.01;***p<0.001 vs. control subjects

thyrotropin-releasing hormone (TRH), luteinizing hormone-releasing hormone, neurotensin, bombesin, and anorexigenic peptide (Cummins and O'Connor 1998). To our knowledge, there are no reports that describe serum changes of these peptides in CIR. However, regarding the plasma clearance rate of TRH, contradictory results have been reported (Iversen et al. 1990; Duntas et al. 1993). While Iversen et al. (1990) reported that in patients with chronic liver disease plasma clearance rate of TRH was significantly greater than in normal subjects, indicating indirectly a possible increased PGLUAP activity in the tissue compartment and/or in plasma,

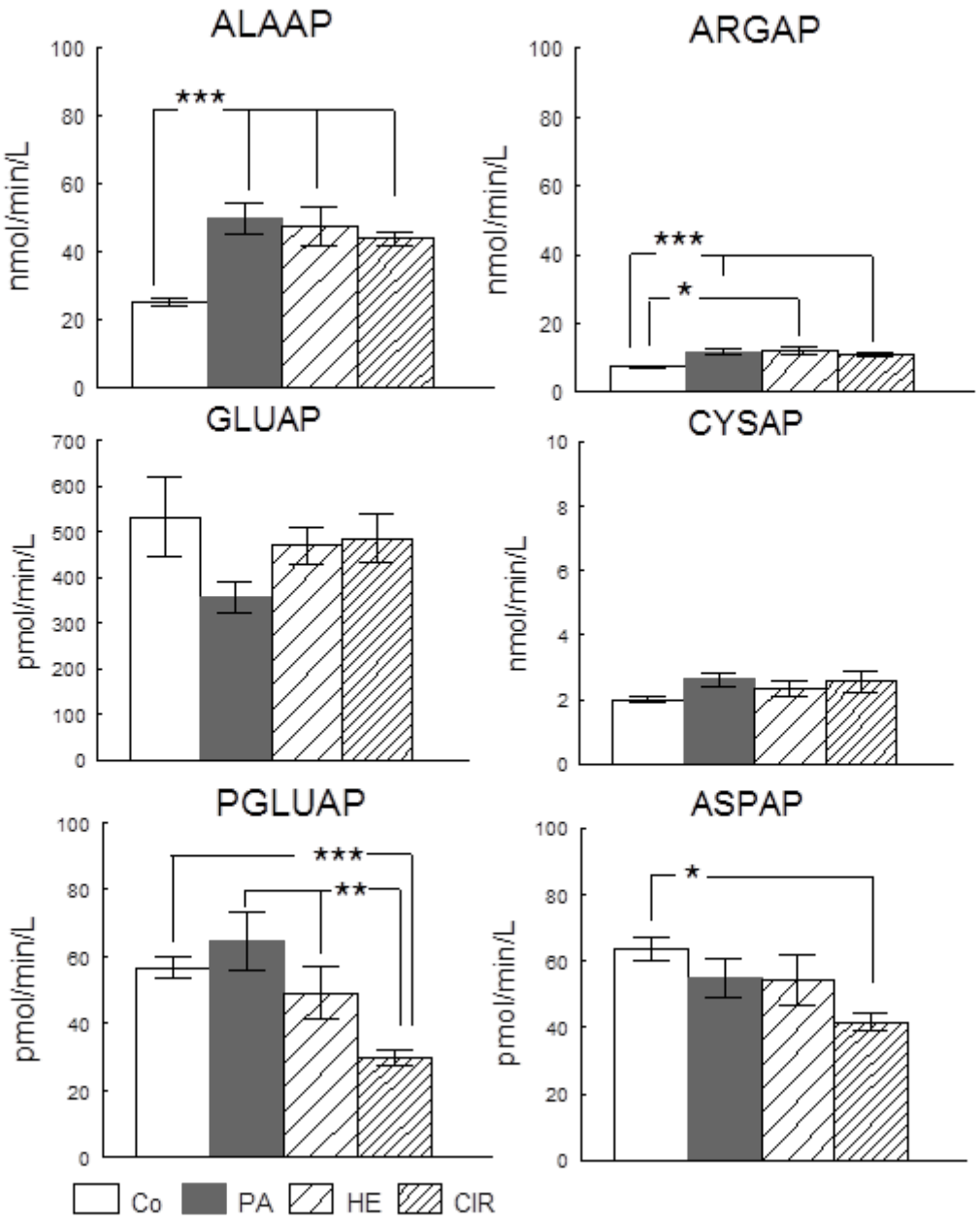

Fig. 1. Serum alanyl- (ALAAP), arginyl- (ARGAP), glutamyl- (GLUAP), cystinyl- (CYSAP), pyroglutamyl- (PGLUAP) and aspartyl- (ASPAP) aminopeptidase activities in control subjects (Co) and in patients with pancr;eatitis (PA), hepatitis (HE) and liver cirrhosis (CIR). Values represent mean ± SEM of aminopeptidase activities expressed as nmol or pmol of the corresponding aminoacyl-β-naphthylamide hydrolyzed per minute per liter of serum. * p<0.05, ** p<0.01, *** p<0.001

Duntas et al. (1993) observed that the inactivation of TRH was less rapid in the presence of blood extract from cirrhotic patients than that from normal subjects or patients with pancreatitis, suggesting that the activity of serum TRH-degrading enzymes was reduced in liver disease. However, to our knowledge, there are no studies that specifically determined PGLUAP activity in the serum of patients with CIR. Therefore, due to the specificity of the results of PGLUAP observed in the present study, we could suggest that the reduction of this enzyme in serum might be considered as a potential candidate to be included in a combination of markers for the diagnosis of CIR.

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