

Heparin-binding EGF-like growth factor in human serum. Association with high blood cholesterol and heart hypertrophy

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Abstract

Heparin-binding epidermal growth factor-like growth factor (HB-EGF) belongs to the EGF family of growth factors which are ligands of the ErbB receptors. Studies in animals suggest the role of HB-EGF in several pathogenic processes such as atherosclerosis and heart hypertrophy. Here, we set up an assay to measure HB-EGF in human serum. Our ELISA determined serum HB-EGF in the range of 0.03–3 nM. It did not cross-react with EGF or with transforming growth factor- α . The mean serum HB-EGF was 0.26 nM (confidence interval: 0.13–0.39) in women and 0.28 nM (confidence interval: 0.09–0.47) in men. In a cohort of 121 healthy volunteers, we identified nine individuals with high serum HB-EGF (above 0.47 nM). These individuals had higher left ventricle mass (determined by Colour Doppler echocardiography) and greater total and low density lipoprotein cholesterol than control. On the basis of our results, we propose that increased serum HB-EGF is associated with heart hypertrophy and elevated blood cholesterol.

Keywords: *body mass index, arterial blood pressure, heart hypertrophy*

Introduction

Heparin-binding epidermal growth factor-like growth factor (HB-EGF) belongs to the EGF family of growth factors (Higashiyama et al. 1991). It is synthesized in many cell types as a membrane-bound precursor protein (Harris et al. 2003). Several matrix metalloproteinases release the soluble active form of this growth factor (Sanderson et al. 2006) which then interacts with target cells. The effects of HB-EGF are mediated by ErbB1 and ErbB4 receptors (Yarden and Sliwkowski 2001).

Increased production of HB-EGF has been implicated in several physiological and pathological processes. It is involved in heart hypertrophy induced by pressure overload, catecholamines or angiotensin II (Asakura et al. 2002); in atherosclerotic plaque growth

(Miyagawa et al. 1995); and in regenerative processes in liver (Kiso et al. 2003), kidney (Homma et al. 1995) and intestine (Pillai et al. 1999). HB-EGF also participates in wound repair after an acute injury (Marikovskiy et al. 1993) and in tumour growth (Raab and Klagsbrun 1997; Fischer et al. 2003). Therefore, the measurement of HB-EGF concentration in human serum may be of clinical relevance.

Few studies have assayed HB-EGF concentration in human fluids. Yamada et al. (1998) described a sandwich-ELISA to measure HB-EGF in plasma samples that required partial purification before the assay. This system was later used by Matsumoto et al. (2002) to study the relationship between HB-EGF and cardiovascular diseases. A simple non-competitive ELISA assay has been used to measure HB-EGF in

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human serum (Keay et al. 2000). Here, we describe a sensitive ELISA to measure HB-EGF in human serum. This assay does not require previous sample processing.

Material and methods

Reagents

Human recombinant HB-EGF (cat. n. 259-HE. Residues Asp⁶³-Leu¹⁴⁸ corresponding to the shed extracellular domain) was obtained from RD Systems (Vitro S.A., Madrid, Spain). The vial (50 µg) was reconstituted in 0.909 ml of PBS containing 0.1% BSA (Sigma-Aldrich, St Louis, MO, USA). Aliquots can be stored at -20°C without loss of activity for more than 1 year. Anti-human HB-EGF monoclonal antibody was obtained from RD Systems (cat. n. MAB259). The vial (0.5 mg) was reconstituted in 1 ml PBS and aliquots were stored at -20°C for at least 1 year without loss of activity. Goat immunoglobulin G (IgG) peroxidase conjugate anti-mouse IgG was obtained from Nordic Immunologicals (Tilburg, The Netherlands). Human recombinant EGF and rat synthetic transforming growth factor-α (TGF-α) were purchased from Sigma-Aldrich (Tres Cantos, Madrid, Spain).

Volunteers

Healthy volunteers (men and women from 35 to 55 years old) were recruited from patients who attended the medical centre (Centro Internacional de Medicina Avanzada, Barcelona, Spain) for routine controls or check-ups. Upon the start of the study, blood pressure measurement, 12-derivative electrocardiogram and Colour Doppler echocardiography (CDE) were done on all volunteers. CDE was performed according to Evangelista-Masip et al. (2000). Blood samples for biochemical analysis were obtained after overnight fasting. For HB-EGF ELISA, serum samples were stored at -20°C and assayed in less than 2 weeks.

HB-EGF ELISA

HB-EGF in serum was measured by indirect antigen-inhibited ELISA. The procedure was based on that previously described for mouse EGF (Grau et al. 1994). One and a half nanograms of HB-EGF (in 0.1 ml PBS) were adsorbed (overnight at 4°C in humidified chamber) onto ELISA plates (96-well EIA/RIA clear flat bottom polystyrene high bind microplate, Corning Inc., Corning, NY, USA) and fixed in mild conditions by adding 0.1 ml of 25% isopropanol in 10% acetic acid for 15 min at room temperature. The plates were then rinsed (three times) with PBS at room temperature and the remaining adsorbent sites were blocked with 5% defatted milk

powder in PBS (30 min at 37°C in a humidified chamber). The plates were then rinsed three times with MTP (0.5% defatted milk powder, 0.1% Tween-20 in PBS) and incubated with 0.05 ml of the primary antibody (mouse antiserum anti-hHB-EGF diluted 1/10,000 in MTP) and 0.05 ml of serum (diluted 1/2 in MTP) or standards (ranging from 3.3 pM to 33.3 nM HB-EGF in MTP) overnight at 4°C in a humidified chamber. After three rinses with MTP, the plates were incubated with 0.1 ml of the secondary antibody (goat-IgG anti-mouse IgG/peroxidase conjugate diluted 1/10,000 in MTP) for 90 min at 37°C in a humidified chamber. The plates were finally rinsed three times with MTP and developed with 0.1 ml of OPD solution (0.4 mg/ml *o*-phenyldiamine, 60 ppm H₂O₂ in 0.15 M citrate buffer pH 5.0) for 20 min at room temperature and the reaction was stopped with 0.05 ml of 2.5 M HCl. Absorbance was measured at 492 nm. Both standards and samples were run in triplicate.

HB-EGF immunoprecipitation and Western blotting

Commercial HB-EGF (275 ng) or serum samples (0.1 ml) were diluted in lysis buffer (Pareja et al. 2003) supplemented with 1% Nonidet P-40, to a final volume of 0.2 ml. They were incubated overnight at 4°C with 2 µg of anti-HB-EGF and the antigen-antibody complex was precipitated by adding 0.025 ml of protein G-Sepharose (Sigma-Aldrich St Louis, MO, USA). After 2 h at 4°C, the mixture was centrifuged and rinsed three times in lysis buffer. Western blotting was performed according to standard procedures after running samples on 15% SDS-PAGE. Primary antibody was used at 1/1000 dilution. Secondary antibody was used at 1/5000 dilution.

Results

The HB-EGF assay

The indirect antigen-inhibited ELISA is based on competition between adsorbed and soluble antigen (HB-EGF) for the soluble antibody. Since the amount of the adsorbed antigen and the dilution of the primary antibody are fixed parameters, the amount of antibody bound to the adsorbed antigen depends on the concentration of soluble antigen. As shown in Figure 1, the higher the soluble HB-EGF concentration, the lower the amount of antibody bound to the plate. Our assay system was specific for HB-EGF. Other related growth factors (EGF and TGF-α) did not compete with adsorbed HB-EGF for binding to the primary antibody. The assay system measured HB-EGF concentration in the range of 0.03–3 nM.

The assay gave linear results between 0.01 and 0.04 ml of serum (data not shown). We routinely assayed 25 µl of serum. Sample did not require

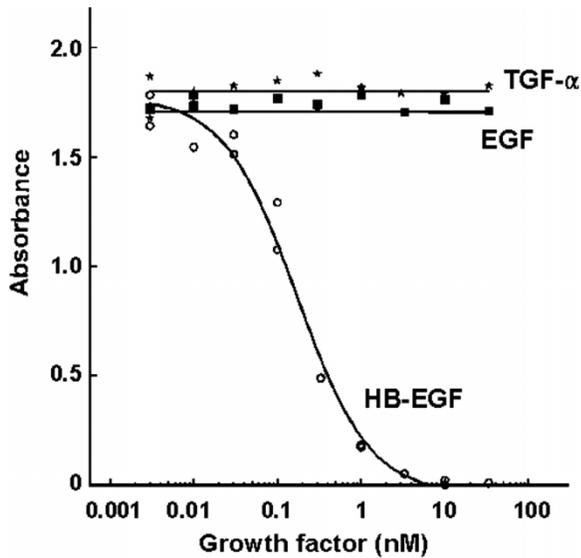


Figure 1. Specificity of the ELISA. Increasing concentrations of human recombinant HB-EGF, human EGF or rat synthetic TGF- α were prepared in MTP and used to compete with adsorbed HB-EGF (0.158 pmol) for binding to murine monoclonal anti-human HB-EGF antibodies. Only HB-EGF produced a dose-dependent displacement of antibodies bound to adsorbed HB-EGF.

previous processing. When we added a known amount of purified HB-EGF to a serum sample, we recovered 98% of the amount added; therefore, the antigen-antibody interaction was not affected by other serum proteins.

Figure 1 shows duplicate values of each standard concentration. The intra- and inter-assay coefficients of variation were 16 ± 2 and $21 \pm 3\%$, respectively. To determine whether the ELISA required fresh samples or tolerated the use of previously frozen samples, we obtained 20 serum samples and measurements of HB-EGF concentration were analysed immediately (fresh samples) or after 15 days at -20°C . HB-EGF concentration was 0.21 ± 0.02 and 0.19 ± 0.01 nM in fresh and stored samples, respectively (difference was not significant as shown by paired Student's *t*-test).

Next, we compared in a western blot assay, the electrophoretic mobility of commercial HB-EGF with that of immunoprecipitated material from the serum sample with the highest HB-EGF concentration. As shown in Figure 2A, commercial HB-EGF (immunoprecipitated (lane 2) or not (lane 1)) showed a major band of 9.5 kD. An identical band was obtained in immunoprecipitated material from the human serum sample (lane 3). Immunoprecipitation made the HB-EGF concentration (ELISA) in the serum sample undetectable (Figure 2B). Therefore, HB-EGF measured by the ELISA in serum samples corresponds to the mature ADAM-shaded form of this growth factor.

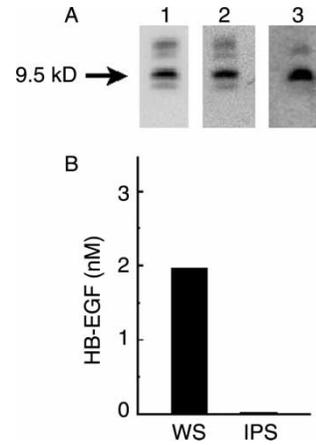


Figure 2. The HB-EGF measured in serum samples correspond to the mature extracellular shed domain. (A) Commercial HB-EGF (immunoprecipitated (lane 2) or not (lane 1)) and immunoprecipitated HB-EGF from the serum sample with the highest concentration determined in the ELISA (lane 3), were run on 15% SDS-PAGE. HB-EGF was detected by Western blot. (B) HB-EGF concentration was determined by ELISA in the serum sample before immunoprecipitation (whole serum: WS) and in the supernatant after immunoprecipitation (immunoprecipitates serum: IPS). Results are the mean of triplicate values.

Serum HB-EGF in healthy volunteers

We recruited a cohort of 121 healthy volunteers (18 women and 103 men without previously diagnosed diseases). The serum HB-EGF values had a Gaussian distribution (data not shown) without significant differences between sexes: 0.28 ± 0.02 (confidence interval: 0.09–0.47) and 0.26 ± 0.02 (confidence interval: 0.13–0.39) nM for men and women, respectively.

HB-EGF is associated to atherosclerosis and is involved in heart hypertrophy. Thus, we divided the cohort into two groups. The first group contained individuals with normal HB-EGF concentration in serum, within the confidence interval, which served as a control. The second group contained those individuals with high HB-EGF concentration in serum: above confidence interval limits. The control group (C) comprised 112 individuals and the high HB-EGF group (H) 9. Mean serum HB-EGF in H was almost threefold higher than in C (Figure 3). There were no age differences between groups (46 ± 1 and 46 ± 3 years in C and H, respectively). Neither were there differences in body mass index (BMI) and arterial blood pressure (ABP) between the groups (Figure 3). However, left ventricle mass (LVM) measured by CDE was significantly higher in H (Figure 3).

Plasma glucose and triglycerides did not differ between individuals with high or normal serum HB-EGF (Figure 4). On the contrary, both total and LDL cholesterol were slightly increased in individuals with high serum HB-EGF (Figure 4). Parameters related to liver (γ -GT and transaminases) and renal (urea and creatinine) function were similar in the two groups (Figure 5).

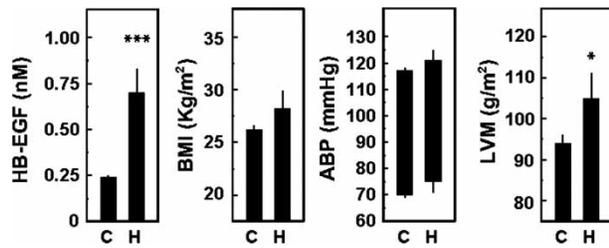


Figure 3. High serum HB-EGF is associated with heart hypertrophy but not with BMI or ABP. Individuals were grouped into those with normal serum HB-EGF (C: HB-EGF < 0.47 nM) and those with high serum HB-EGF (H: HB-EGF \geq 0.47 nM). The figure shows the serum HB-EGF concentration, BMI, arterial blood pressure (ABP, systolic/diastolic) and LVM determined by CDE. Bars correspond to the mean \pm SE. The significance of the differences vs. the control group was determined by Student's *t*-test. * $p < 0.05$; *** $p < 0.001$.

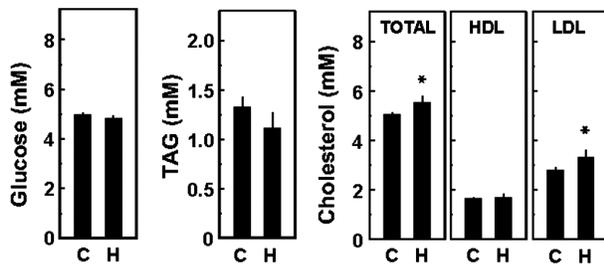


Figure 4. High serum HB-EGF is associated with total and LDL cholesterol but not with plasma glucose or triglycerides. Individuals were grouped into those with normal serum HB-EGF (C: HB-EGF < 0.47 nM) and those with high serum HB-EGF (H: HB-EGF \geq 0.47 nM). The figure shows plasma glucose, triglycerides (TAG) and total, HDL and LDL cholesterol. Bars correspond to the mean \pm SE. The significance of the differences vs. the control group was determined by Student's *t*-test. * $p < 0.05$.

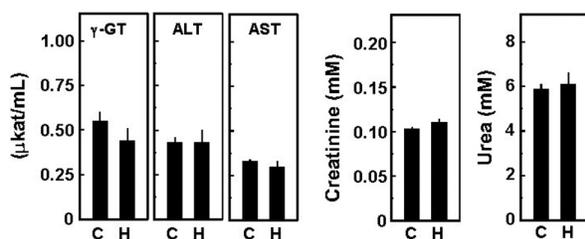


Figure 5. High serum HB-EGF is not associated with functional parameters of liver or kidney. Individuals were grouped into those with normal serum HB-EGF (C: HB-EGF < 0.47 nM) and those with high serum HB-EGF (H: HB-EGF \geq 0.47 nM). The figure shows plasma-glutamyltranspeptidase (γ GT), alanine (ALT) and aspartate (AST) transaminase activities and plasma creatinine and urea. Bars correspond to the mean \pm SE. The significance of the differences vs. the control group was determined by Student's *t*-test.

Discussion

Many studies have addressed HB-EGF function in animal models (review in Iwamoto and Mekada (2006)). However, few have been performed in humans, partly because an appropriate assay system

for HB-EGF in serum or plasma samples is not available. Here, we describe a simple and sensitive ELISA to measure the HB-EGF in human serum. In a cohort of 121 healthy individuals, we observed that high HB-EGF in serum was associated with increased LVM and higher plasma LDL cholesterol. Although preliminary, these results indicate that it will be worth to further explore the relationship between serum HB-EGF and heart diseases.

Our assay system is based on commercial reagents. Therefore, it can be easily set up in research laboratories and it is simple because it does not require sample processing. Our ELISA system described here is sensitive (it can measure HB-EGF in serum at concentrations as low as 0.03 nM) and specific (it does not cross-react with other members of its growth factor family (EGF and TGF- α)).

The first attempt to measure HB-EGF in human plasma was reported by Yamada et al. (1998). They described a sandwich ELISA linked to an amplification system. Their assay required previous partial purification of HB-EGF from plasma samples. Key et al. (1997) reported a simpler method for measuring HB-EGF in urine which involved the adsorption of urine directly onto the wells. This assay system was used in later studies (Chai et al. 2000; Zhang et al. 2005).

In our assay, the mean HB-EGF concentration in human serum was 0.28 and 0.26 nM in men and women, respectively. Yamada et al. (1998) reported a much lower concentration (1.7 pM) although in a later report by the same laboratory (Matsumoto et al. 2002), a higher concentration was found (7 pM). The requirement of previous HB-EGF partial purification from samples may explain the lower concentrations described in these reports. The type of human fluid sampled (plasma vs. serum) does not explain this difference. For other growth factors, platelets accumulate in a large amount, thereby explaining differences in concentration when measured in plasma and serum (Mattila et al. 1988; Pesonen et al. 1989). However, to the best of our knowledge, there are no reports showing the occurrence of HB-EGF in human platelets. In addition, we did not observe differences between HB-EGF concentration measured in plasma (heparin, citrate or EDTA as anticoagulants) and in serum (data not shown).

In vitro studies suggest the role of HB-EGF in the progression of atherosclerotic plaque (Miyagawa et al. 1995; Peoples et al. 1995; Nakata et al. 1996). In keeping with these studies, patients with coronary artery disease show an increase in plasma HB-EGF concentration (Matsumoto et al. 2002). Our cohort did not include patients with this disease; however, we observed that high serum HB-EGF is associated with high total and LDL cholesterol in plasma, the major risk factor for coronary artery disease. Matsumoto et al. also found a significant positive

correlation ($r = 0.48$) between HB-EGF in plasma and BMI. We did a similar correlation study in our cohort (data not shown), but obtained a lower and non-significant correlation coefficient ($r = 0.13$). It should be noted that our cohort included individuals with BMI values between 20 and 39. Nineteen of them had a BMI equal to or higher than 30.

The relevance of HB-EGF in the induction of heart hypertrophy was established by Asakura et al. (2002) in studies using inhibitors of the metalloproteinases responsible for processing the membrane-anchored HB-EGF precursor. Our results indicate that in human serum a high HB-EGF concentration is associated with greater LVM, as determined by CDE. Indeed, new studies are required to establish the relationship between serum HB-EGF levels and the progression of heart hypertrophy and eventually heart failure. But, since it was described that overexpression of HB-EGF exacerbates remodelling after myocardial infarction (Ushikoshi et al. 2005), we suggest that the measurement of HB-EGF in serum samples may be of interest in the control of heart diseases. However, the ErbB system is quite complex and several ligands can be released by the same metalloproteinases. Therefore, a complete analysis of ErbB ligands will be required for the understanding of the contribution of the ErbB system to heart disease.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Note

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