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# Different Pairs of Monoclonal Antibodies Detect Variable Amounts of Soluble Endoglin in Human Blood Plasma

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## Abstract

Soluble endoglin is produced as a result of extracellular domain cleavage of full-length membrane molecules. Numerous studies of recent years have shown that its excessive production is associated with vascular pathology, tumor growth and preeclampsia development. In this study we developed and explored ten sandwich-ELISA systems, based on monoclonal antibodies (MAbs) produced in our laboratory, to detect soluble endoglin in blood plasma. Here we demonstrated that values of endoglin concentration determined in the same samples varied greatly depending on pairs of MAbs used. Some measurements were up to two orders of magnitude higher compared with widely used commercial kit. Western blot analysis revealed four fractions of the antigen precipitated from plasma. Our data suggest that this molecular heterogeneity of soluble endoglin may lead to the pronounced differences in estimates of its concentration made using different sandwich-ELISA systems. Nevertheless, all studied systems revealed increment of endoglin content in plasma of pregnant women compared with healthy donors as well as its substantial production in preeclampsia patients.

**Keywords:** Soluble endoglin; CD105; Monoclonal antibody; Sandwich-ELISA; Pregnant women; Preeclampsia

**Abbreviations:** ELISA: Enzyme-linked immunosorbent assay; Eng-NS0: Recombinant endoglin molecules produced by mouse myeloma cells NS0; MAbs: Monoclonal antibodies; PAGE: Polyacrylamide gel electrophoresis; TBS: Tween Tris-buffered saline supplemented with Tween 20; TMB: Tetramethyl-benzidine solution.

## Introduction

Endoglin (CD105) is a transmembrane homodimeric glycoprotein that mainly expressed on endothelial cells [1], syncytiotrophoblast [2], and mesenchymal stem cells [3]. Initially it was classified as a co-receptor for TGF-β1 and TGF-β3 [4]. Later studies have established it is also involved in the reception of activin A, BMP-2, BMP-7, BMP-9, and BMP-10 [5-7]. Endoglin is up-regulated on the surface of activated endothelial cells [8] and contributes to angiogenesis in embryonic development [9] and cancer [10].

Endolgin in soluble form is found in human blood plasma [11] and cell culture media of endothelial cells [12]. These molecules are released from the cell surfaces as a result of extracellular domain cleavage by matrix metalloproteinase-14 [12]. Excessive soluble endoglin production is observed in cancer patients and pregnant women who have preeclampsia and correlates with the severity of the diseases. Thus, endoglin concentration is considered as a valuable diagnostic feature for the prognosis of cancer [13] and the risk estimation of preeclampsia [14]. Growing amount of evidences indicate its involvement into the pathogenesis of atherosclerosis [15]. Thus, sensitive methods for estimation of soluble endoglin concentration have wide range of applications in fundamental and clinical research.

Recently we have generated a new set of hybridomas producing monoclonal antibodies (MAbs) against human endoglin [16]. In this study we developed and explored ten sandwich-ELISA systems, based on these MAbs, for the measurement of soluble endoglin in blood plasma samples. Unexpectedly, antigen concentrations determined by different pairs of MAbs varied greatly. Some of them were up to two orders of magnitude higher compared with a reference kit. Western blot analysis of immunoprecipitated endoglin revealed molecular heterogeneity of the antigen. Four endoglin fractions having various molecular weights were detected. We suggest this heterogeneity of endoglin may lead to the pronounced differences in estimates of its concentration made using different sandwich-ELISA systems. Nevertheless, all pairs of MAbs detect differences in the antigen levels in blood of healthy donors, healthy pregnant women, and women with preeclampsia.

## Materials and Methods

### Monoclonal antibodies

The generation of ten MAbs is described in our previous article [16]. Two new reagents, called 4G8 and 5F1, were produced and characterized in the same way. MAb 4F4 is referred to as 4E4 in this study. Antibodies were purified from ascitic fluids of F1(SJL/J ×

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Balb/c) mice by protein G affinity chromatography and precipitated by ammonium sulfate. Peroxidase labeling of MAbs was performed according to the protocol described in Ref. [17].

### Cell cultures

Human hepatocellular carcinoma cell line HEP G2 [18] was obtained from the cell culture collection at the Institute of Cytology, Russian Academy of Sciences (Saint-Petersburg, Russia). Cell cultures were grown in DMEM medium supplemented with 5% fetal calf serum (BIOLOT).

### Blood plasma samples

Blood plasma samples from healthy donors, healthy pregnant women, and women who had preeclampsia (36–38 weeks of pregnancy) were collected after informed consent in the Research Institute for Obstetrics, Gynecology and Reproductology named after D.O. Ott (St. Petersburg, Russia). Heparin was used as anti-coagulant. Pooled plasma samples were produced by mixing equal volumes of individual samples obtained from ten randomly selected healthy donors or women with preeclampsia.

### ELISA

Tris-buffered saline supplemented with 0.5% Tween 20 (TBS-Tween) was used for reagents dilution and plate washing in all ELISA tests. Tetramethyl-benzidine solution (TMB, Thermo Scientific) was used as a substrate-chromogen solution. Enzyme reaction was stopped by adding 1N sulfuric acid. Optical density was measured at 450 nm.

Competitive ELISA on HEP G2 cells was used for the investigation of epitope co-localization on the antigen. Detailed description of plate preparing procedures is provided in our previous work [16]. The mixture of peroxidase-labeled and unlabeled MAbs (1:1) was added to the cell-containing wells and incubated for 2 h at +37°C. Working dilutions of labeled reagents were selected so that their binding with the antigen could be completely inhibited by 10 μg/ml of identical unlabeled MAbs. Optical densities in the wells containing TBS-Tween instead of unlabeled MAbs were assumed as the absence of inhibition and set as 0%. Optical densities in the wells containing identical labeled and unlabeled MAbs were considered as complete inhibition and set as 100%. Inhibition rates of not-matching MAbs were calculated based on optical densities in corresponding wells and these two values. All experiments were made in four replicates and median values were used for the analysis. Epitopes recognized by two MAbs were considered as overlapping if their inhibition were higher than 85%, otherwise they were assumed as distant.

Quantitative determination of endoglin in blood plasma samples was carried out using sandwich-ELISA. Unlabeled MAbs diluted in carbonate-bicarbonate buffer (10 μg/ml) were adsorbed on the solid phase for 18 h at +4°C. Endoglin-containing samples were incubated in wells for 1 h at +37°C. Formed immune complexes were detected using a second, peroxidase-labeled MAbs. Recombinant soluble endoglin produced by mouse myeloma cells NS0 (1097-EN-025, R&D SYSTEMS) was used as a calibrator.

A reference system, commercial ELISA kit for detection of soluble endoglin (Human Endoglin/CD105 Quantikine ELISA Kit, R&D Systems), was used according to the manufacturer's instructions.

### Immunoprecipitation of soluble endoglin

Immuoprecipitation of soluble endoglin from plasma samples was carried out as described in Ref. [17]. Briefly, anti-endoglin MAb 2C8 was immobilized on cyanogen bromide-activated-sepharose (GE Healthcare). Plasma samples were diluted 3-fold in PBS and loaded on MAb-containing carrier. Molecules retained on the column were eluted by citrate-phosphate buffer (pH 3.6). Endoglin-containing eluates were concentrated using Vivaspin concentrators with molecular-weight cut-off of 30 kDa (Z614637, SARTORIUS). Solutions obtained on each step were collected and tested for endoglin content.

### SDS-PAGE and western blot

Endoglin molecules obtained from the affinity column were analyzed by SDS-PAGE on 7.5% acrylamide gels under reducing conditions and electrophoretically transferred onto nitrocellulose membranes. Filters were blocked with 2.5 mg/ml casein in TBS-Tween for 30 min at room temperature with constant agitation. Primary anti-endoglin MAbs 2E1 (5 μg/ml) or 4B7 (10 μg/ml) were added to the membranes and incubated overnight at +4°C. The secondary reagent, polyclonal goat anti-mouse Ig antibody conjugated with horseradish peroxidase, was added in the selected dilution (1:30 000). Incubation was carried out for 1 h at room temperature with constant agitation. Both specific reagents were diluted on the blocking buffer. The presence of peroxidase activity on membranes was revealed by TMB solution (T0565, SIGMA). Molecular weights of endoglin fragments were estimated using PageRuler pre-stained protein ladder (26616, THERMO FISHER SCIENTIFIC). Densitometric analysis of western blot membranes was made using ImageJ software [19].

### Statistical analysis and data visualization

Statistical analysis and data visualization were performed using the R programming language (version 3.2.2) and graphic packages ggplot2 and cowplot.

## Results

In our laboratory we generated and described a new panel of MAbs against human endoglin. Ten of these reagents (1B4, 2C8, 3G2, 4C9, 4E4, 4G8, 5B6, 5F1, 5G7, 5H7) are specific to conformational epitopes and recognize the antigen expressed on the cell surfaces. In contrast, MAbs 2E1 and 4B7 bind two distant linear epitopes that are normally hidden on the membrane molecules. All reagents recognize recombinant endoglin molecules produced in *E. coli* or mammalian cells [16].

At the first step of the study we established the spatial localization of conformational epitopes recognized by MAbs. For this purpose competitive ELISA on endoglin-expressing HEP G2 cells was used. Each antibody was conjugated with horseradish peroxidase. Labeled and unlabeled reagents were mixed in equal proportions and tested on fixed cells. MAbs demonstrating high inhibition rates were considered as recognizing spatially overlapping epitopes (Figure 1A). Obtained results clearly showed that three antibodies (3G2, 4C9, and 4G8) recognized isolated parts of the endoglin molecule, while the remaining seven reagents were specific to a cluster of partially overlapping epitopes (Figure 1B).

Next we tested all combinations of MAbs displaying low reciprocal inhibition activities as components of sandwich-ELISA systems. Unlabeled reagents were adsorbed on the solid phase while peroxidase-conjugated MAbs served as signal-developing agents. Serial dilutions (from 100 to 0.1 ng/ml) of commercial recombinant endoglin produced in mouse myeloma cells NS0, Eng-NS0, were tested using each pair of MAbs. The most sensitive combinations of the reagents included eight out of twelve MAbs and were able to detect as little as 0.1-1.0 ng/ml of

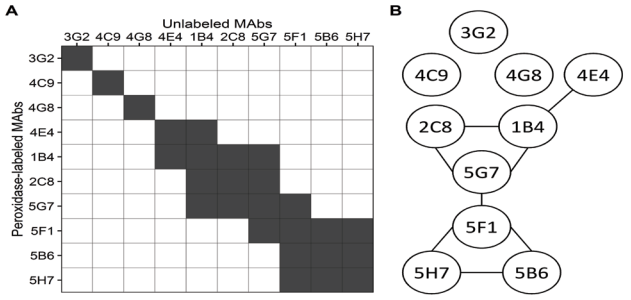


**Figure 1:** Relative localization of epitopes recognized by MAbs revealed in competitive ELISA on endoglin-expressing HEP G2 cells. (A) Mutual inhibition of binding of the peroxidase-labeled MAbs by the unlabeled reagents. Filled squares indicate strong inhibition (>85%) of the color reaction. (B) Node plot of the spatial relationship between epitopes recognized by the MAbs. Lines connect overlapping epitopes.

the antigen. All pairs including labeled or unlabeled MAbs 2E1, 3G2, 4B7 or 4G8 demonstrated weak reactivity to Eng-NS0 in solution.

For the further step we chose ten most sensitive pairs of MAbs and explored their abilities to detect soluble endoglin in pooled blood plasma samples obtained from healthy donors and women with preeclampsia. Commercial ELISA kit for human endoglin was used as a reference system. In each test calibration curves were generated by titrating Eng-NS0. Unexpectedly, estimates of endoglin concentration in the same samples varied greatly depending on the combinations of capture and signal-developing antibodies used (Figure 2). The pair of MAbs consisting of adsorbed on the solid phase 4E4 and peroxidase-labeled 4C9 (4E4-4C9) detected the highest quantities of soluble endoglin. Measured values were almost 100 or 50 times higher than ones estimated in the commercial kit in the samples obtained from healthy donors and women with preeclampsia, respectively. Other combinations of immobilized and labeled MAbs revealed lower levels of the antigen but still much higher compared with the reference system.

In order to verify antigenic specificity of constructed ELISA systems we performed immunoprecipitation of soluble endoglin from the pooled plasma samples using MAb 2C8 developed in our laboratory. Every ELISA system and the reference kit produced strong color reactions when intact or eluate samples were tested, while only background staining was revealed in wells containing flow-through solutions. Quantification of endoglin in the eluates showed that approximately 90% of the antigen was recovered from the plasma. This experiment proved identical antigenic specificity of all ELISA system based on MAbs developed in our laboratory and the commercial kit.

Next we analyzed molecules precipitated from plasma in western blot under reducing conditions. Both samples were prepared in such a way that they contained 200 or 2.6 ng of endoglin estimated by the 4E4-4C9 system or the reference kit, respectively. Another four lanes contained 200, 100, 50 or 25 ng of the calibrating reagent, Eng-NS0. Antigen molecules were revealed by MAbs 2E1 or 4B7 specific for a linear epitope of endoglin. As shown in Figure 4 blood plasma contained up to four different antigen fractions. The band with the highest molecular weight (90 kDa) was detected only in the sample obtained from women with preeclampsia and most likely corresponded to full-length endoglin molecules. Fractions A had the same molecular weight (81 kDa) as Eng-NS0, which include only extracellular domain of the antigen. Therefore, molecules from these fractions might have had similar structure. The remaining two bands apparently contained shorter fragments. Approximately three quarters of the antigen in both samples were concentrated in bands B (70 kDa) and up to 15% in bands C (62 kDa). Densitometric analysis showed that MAb 2E1 revealed 140 or 230 ng of the antigen in lanes containing endoglin molecules precipitated from plasma of healthy donors or women with preeclampsia, respectively. Western blot analysis using MAb 4B7 confirmed these results. Thus, these data demonstrated molecular heterogeneity of endoglin existing in blood (Figure 3).

Finally, we estimated endoglin concentrations in three sets of individual blood plasma samples obtained from healthy donors, healthy pregnant women, and women with preeclampsia. Again, the most pronounced differences were observed between the measurements obtained by the 4E4-4C9 system and the reference kit. They ranged from 25 to 150 times. Nevertheless, all systems detected differences in the antigen levels between groups of donors. Endoglin concentrations in blood plasma of healthy donors were slightly lower compared with pregnant women, while patients with preeclampsia had much higher antigen levels. Here we illustrate our data comparing the 4E4-4C9 system and the reference kit (Figure 4A, Table 1). However, the measured values in different systems demonstrated various correlation levels within three sets of samples (Figure 4B). Thus, they poorly correlated within the group of healthy donors (Pearson's correlations coefficient $\rho$=0.293, p=0.33). Some samples demonstrated almost equal quantities of the antigen when assessed in the commercial kit and up to 3-fold differences when the 4E4-4C9 system was used. Meanwhile

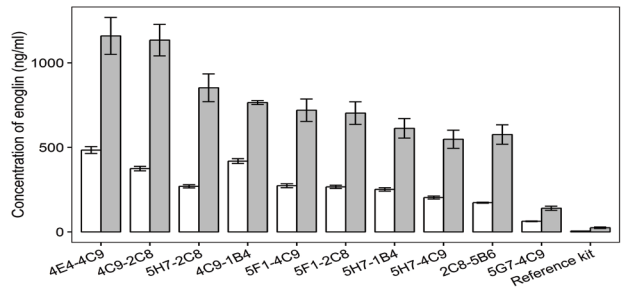


**Figure 2:** Different sandwich-ELISA systems detected variable amounts of soluble endoglin in pooled blood plasma samples obtained from healthy donors (empty bars) and women with preeclampsia (filled bars). Codes of capture and signal-developing reagents are shown below the histogram. Commercially available ELISA kit was used as the reference. All measurements were made in triplicates using Eng-NS0 as a calibrating reagent. Means and standard deviations are shown.

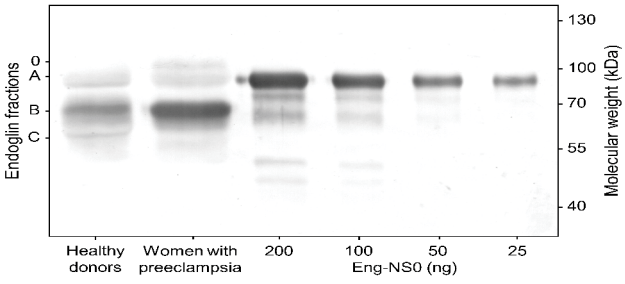


**Figure 3:** Western blot analysis revealed molecular heterogeneity of endoglin from blood plasma of healthy donors and women with preeclampsia. The antigen was precipitated from plasma by MAb 2C8. Tested samples contained 200 or 2.6 ng of endoglin estimated using the 4E4-4C9 system or the reference kit, respectively. The calibrating reagent, Eng-NS0, was added in the last four lanes. Electrophoretic separation was carried out by SDS-PAGE with 7.5% acrylamide gel under reducing conditions. MAb 2E1 detects four fractions of endoglin labeled as 0, A, B, and C.

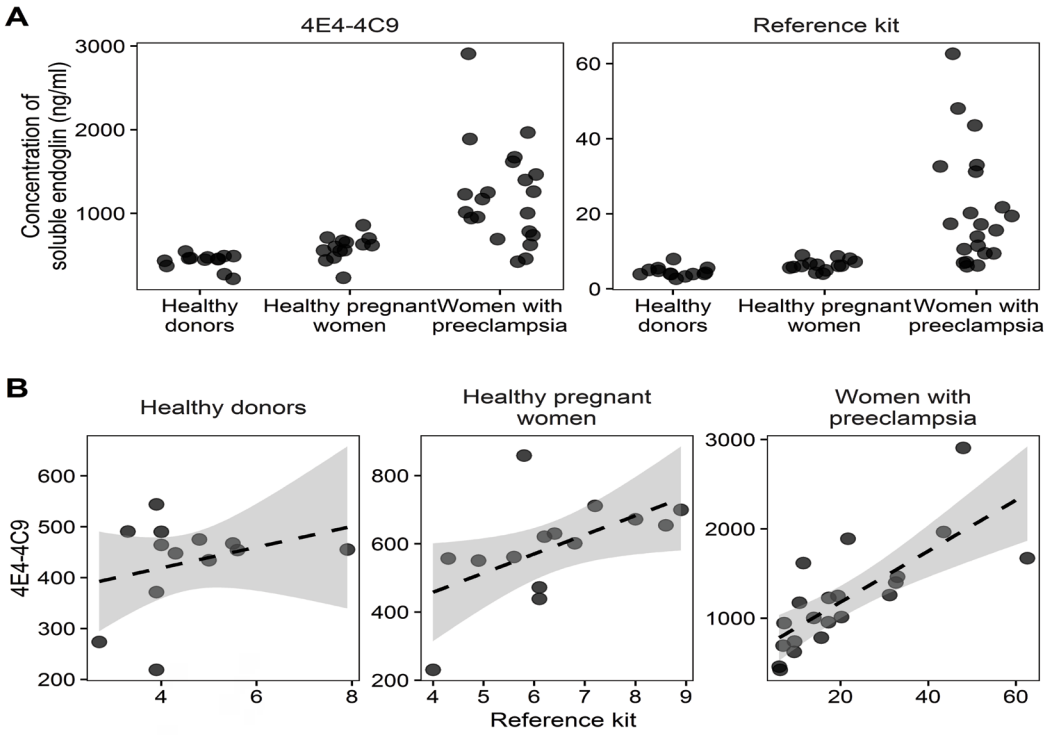


**Figure 4:** Soluble endoglin concentrations in individual blood plasma samples estimated using the 4E4-4C9 system and the reference kit. (A) Both systems detected increment of soluble endoglin concentration in blood plasma of pregnant women compared with healthy donors and substantial production of the antigen in blood of women with preeclampsia. (B) The values (ng/ml) measured in two systems demonstrated different levels of correlation within three sets of samples. Regression lines and their 95% confident intervals (grey areas) are shown.

| Group of donors | Number of samples | Mean ± SD | |
|---|---|---|---|
| | | 4E4-4C9 | Reference kit |
| Healthy donors | 13 | 429.6 ± 90.8 | 4.5 ± 1.3 |
| Healthy pregnant women | 14 | 589.8 ± 147.2 | 6.3 ± 1.5 |
| Women with preeclampsia | 20 | 1212.3 ± 583.9 | 21.1 ± 15.4 |

**Table 1:** The average contents of soluble endoglin in plasma samples obtained from three groups of donors.

estimates of endoglin concentration became more correlated in plasma of healthy pregnant women ($\rho$=0.56, p=0.04) and even more in samples obtained from women with preeclampsia ($\rho$=0.753, p<0.001). The same trend was observed when measurements made using any others pairs of MAbs were considered.

## Discussion

In this study we explored ten sandwich-ELISA systems aimed for the detection of soluble endoglin. Competitive ELISA on HEP G2 cells revealed pairs of MAbs which demonstrated undesirable mutual inhibition when they interact with the antigen simultaneously. Among the remaining combinations of reagents we identified the most sensitive pairs of MAbs which could detect recombinant soluble endoglin at concentrations as little as 0.1-1.0 ng/ml. Unexpectedly, estimates of endoglin content in blood plasma made by selected ELISA systems varied greatly and differed up to two orders of magnitude. Endoglin depletion from blood plasma samples by MAb 2C8 confirmed identical antigenic specificity of constructed ELISA systems and the reference kit. This result along with our previous data [16] supported strict antigenic specificity of generated antibodies.

Western blot analysis of endoglin precipitated from blood plasma revealed molecular heterogeneity of the antigen. Peptide fragments of various lengths formed four distinct bands with molecular weights ranging from 62 to 90 kDa. We suppose that molecules with the highest molecular weight correspond to full-length endoglin and may come from CD105+ microparticles circulating in blood [20]. Fragments of 81 kDa most likely consisted of extracellular domain of endoglin. Peptides of this size were previously isolated from culture media of human umbilical endothelial cells. The authors of the study showed that MMP-14 was responsible for their cleavage from the cell surfaces [12]. The origin of shorter fragments is less clear. Attempts to identify other enzymes involved in endoglin cleavage were not successful [21,22]. Nevertheless, Venkatesha et al. showed the presence of soluble endoglin of 65 kDa in sera of healthy pregnant women and patients with preeclampsia [11]. This fragment most like correspond to the fraction B observed in this study. Even shorter endoglin peptides (45 kDa) were found in culture media of bone marrow-derived HS-5 cells [23]. Here we were unable to detect fragments of this size in blood plasma. Instead fragments of 62 kDa were found.

There is evidence that interaction of soluble endoglin with its natural ligands may provide additional heterogeneity. Thus, the antigen in blood could be found as a component of stable complexes antigen with BMP-9 and BMP-10 in solution [6]. We believe that endoglin molecules containing aggregation-prone ZP domains may form even larger complexes including other proteins. This assumption is partially supported by the study of Li et al. who demonstrated the presence of large endoglin complexes (125-200 kDa) in blood plasma [24,25].

Our data suggest that molecular heterogeneity of soluble endoglin may lead to the pronounced differences in estimates of its concentration made by different sandwich-ELISA systems. Different pairs of MAbs probably do not detect all endoglin fractions because their specific binding sites are missing or hidden by bound proteins in some of them. Nevertheless, all studied ELISA systems revealed increment of endoglin content in plasma of pregnant women compared with healthy donors as well as its substantial production in preeclampsia patients. At the same time correlation between the measurements made within the group of healthy donors was significantly lower compared with other two groups. This might indicate a greater heterogeneity of the antigen in their blood.

Our findings also raise a question about matching endoglin fractions from blood with their biological activity and underlying physiological processes. It has been reasonably proposed that shorter endoglin molecules and molecules bound with other proteins may lose their biological activity [9,11]. In addition, production of endoglin fragments of various lengths may reflect different physiological and pathological processes. Thus, we admit that ELISA systems detecting certain endoglin fractions might have greater prognostic values then the others.

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