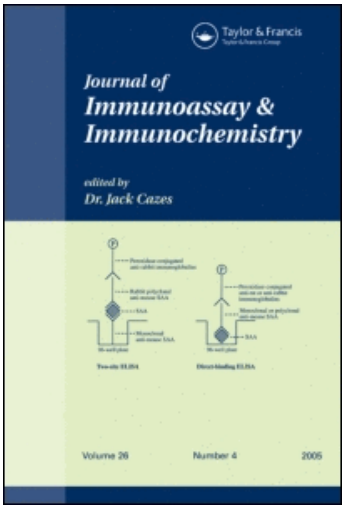

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Development of an Elisa for Quantification of Human Protein S in Cell Culture Fluids Using Commercial Polyclonal Antisera

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DEVELOPMENT OF AN ELISA FOR QUANTIFICATION OF HUMAN PROTEIN S IN CELL CULTURE FLUIDS USING COMMERCIAL POLYCLONAL ANTISERA

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ABSTRACT

An enzyme-linked immunosorbent assay (ELISA) was developed to measure protein S antigen released into cell culture fluids. We used readily available commercial polyclonal antisera to develop the assay. This assay was sensitive with a detection limit of about 0.086 ng/ml. Between-assay precision (coefficient of variation) at levels of 0.2, 1.1, and 13.9 ng/ml was 14%, 15%, and 11% respectively. Specificity and accuracy were demonstrated from the use of: 1) culture fluids from 3-primary endothelial cell cultures and 7-cell lines known to constitutively produce protein S; 2) 2-cell lines not synthesizing protein S; and 3) from selected samples of normal and protein S deficient plasma. The ELISA described here was about 12-fold more sensitive and 40-fold more cost effective when compared to a commercial ELISA kit. Thus the assay provided a sensitive, specific, precise and economical method useful for the measurement of the nanogram amounts of protein S commonly encountered in cell culture fluids. (Key words: Protein S, ELISA, cytokine, endothelial cell)

INTRODUCTION

Protein S is a coagulation regulatory vitamin K-dependent plasma protein. The amount of total protein S in normal plasma, which exists in free and bound forms, is in the range of 19-35 μg/ml (1). Bound protein S is inactive and reversibly and stoichiometrically associated (1:1) with a negative regulator, the complement C4b-binding protein (C4BP), and accounts for about 60% of total plasma protein S (2,3). Free protein S functions as a cofactor for activated protein C (4,5), a zymogen derived serine protease, which inactivates the thrombin generating active factors Va and VIIIa (5-7), thus inhibiting coagulation. Recently, protein S was shown to also act as an antithrombotic agent independently of activated protein C through factor Va binding and inhibition of prothrombinase activity (8). The clinical importance of the protein C/protein S pathway in the regulation of blood coagulation is illustrated by the association of thrombosis with congenital or acquired deficiencies of either protein (9-12).

Accumulating evidence suggests that the expression of endothelial cell derived regulatory proteins involved in hemostasis can be modulated by inflammatory cytokines, thus disrupting their physiological balance and leading to a procoagulant state (13-19). Recent reports of venous thrombosis associated with depressed levels of free protein S in persons with AIDS (20-22) and an awareness of temporal fluctuation in serum levels of inflammatory cytokines e.g. tumor necrosis factor (TNF) and interleukins-1 and -6 as a consequence of AIDS infection or as a result of secondary infection (23) prompted our interest in using an endothelial cell model to

explore the possibility of cytokine mediated protein S regulation. Central to understanding the effects of cytokines on the regulation of gene expression of coagulation regulatory proteins is the ability to measure these gene products with precision, specificity and high sensitivity. This ability is especially important for in vitro studies in which ligand levels may be several thousand fold less than those of the in vivo counterpart. Here we describe the first phase of our study i.e. the development of a highly sensitive, specific, precise, and economical ELISA suitable for measuring nanogram amounts of protein S antigen in a vascular endothelial cell culture model. We used a sandwich format consisting of a coating antibody to capture antigen during sample incubation followed by a second protein S antibody which was detected with a horseradish peroxidase (HRP)-conjugated anti-Ig. The antibodies and reagents used in developing this ELISA were readily available as items of commerce.

MATERIALS AND METHODS

Cell lines and culture fluids

The following cultured human cells and cell lines were used to measure protein S production. For the endothelial cell culture model, we used an SV-40T transfected human microvascular endothelial cell line (HMEC-1) which has been previously characterized (24). Cells were grown in MCDB 131 (Gibco BRL, Grand Island, NY‡) that contained penicillin-streptomycin (50 U/ml and 50μg/ml), 15% fetal bovine serum (Gibco BRL), 10 ng/ml epidermal growth factor and 1

μg/ml hydrocortisone. Human umbilical vein and aortic endothelial cells were obtained commercially (Clonetics, San Diego, CA), and the human dermal microvascular endothelial cells were obtained from Cell Systems Corp, Kirkland, WA and propagated using the manufacturers medium and accompanying instructions. Experiments were performed with HMEC-1 cells at passage levels between 6 and 8 and primary endothelial cells (EC) at passage levels 3-5.

Several histologically diverse tumor cell lines were also examined for protein S production with this ELISA assay. These cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained by the Biological Products Branch, Division of Scientific Services, Centers for Disease Control and Prevention.

To determine the rate of protein S production in culture fluids, either 2.0-cm^2 24-well or 10-cm^2 6-well dishes were plated at a density of 0.5 X 10^5 cells/cm^2; after monolayers were confluent, culture fluids were aspirated, monolayers washed with a balanced salts solution and fresh media added (zero time). Tissue culture fluid samples were harvested at the indicated intervals for protein S measurement, and cell counts were determined. Culture fluids from 4-8 replicate culture wells were added directly to ELISA plate wells without dilution.

Plasma and serum specimens

Selected plasma and serum specimens were used to demonstrate accuracy of protein S measurement and further confirm assay specificity. Paired blood samples were collected by venipuncture in

citrate-dextrose and without anticoagulant and centrifuged 2500 X g for 20 min at 4° C. Platelet-poor plasma or serum was then removed and quick frozen at -70° C until used. A plasma sample rendered deficient in protein S by immunoaffinity chromatography was purchased commercially (American Diagnostica, Inc., Greenwich, CT) and tested for protein S detection by ELISA.

Free protein S in plasma was measured by first removing bound protein S in the C4BP complex using polyethylene glycol 8000 precipitation at a final concentration of 3.75% as previously described (25). Supernatant fluids were then used to measure free protein S. Total protein S in plasma or serum was measured after empirically determining (see Fig. 3) the dilution and incubation time sufficient for dissociation of the protein S-C4BP complex. Sample dilutions were measured in triplicate in the ELISA.

Internal standards or quality control samples were prepared from pooled plasma as dilutions representing high-, mid-, and low-range points on the standard curve after calibration against a purified protein S standard. These samples were sub-tubed and stored -70° C for use in each ELISA.

Antisera and reagents

Polyclonal goat anti-protein S, ammonium sulfate fraction, lots #89-02E, 89-02M and 90-004M, (American Diagnostica) was further purified by fractionation using DEAE Sephadex A50. After column and sample equilibration in 0.1 M Tris-HCl pH 8.3, 0.05 M NaCl, the IgG fall-through was collected and stored at 4° C until used. The IgG concentration was determined from the extinction coefficient $E_{278\ nm}$

= 14.3. A rabbit anti-protein S, IgG isolated by ion exchange chromatography, containing 14 mg/ml of protein and approximately 150 μg/ml of antibody (Lots # 080H4810 and 080H4811) was purchased form Sigma, ImmunoChemicals, St Louis, MO. The HRP-conjugated donkey IgG anti-rabbit Ig (species specific) was purchased from Amersham, Arlington Heights, Il.

Optimal concentrations of the different antibody preparations were empirically determined by titration of each reagent for maximal signal-to-noise response.

Protein S, isolated by barium adsorption and elution followed by immunopurification, was obtained commercially (American Diagnostica) and used as a calibrator in the ELISA developed here and also in a comparative evaluation of a commercial ELISA kit. A Coomassie blue stained preparation in 8% polyacrylamide gel electrophoresis (PAGE) had a single band at approximately 75 kDa and showed no reduction in 2-mercaptoethanol-SDS-PAGE. Protein S concentration was determined using and extension coefficient of $E^{1\%}_{278\ nm} = 9.5$.

ELISA

For protein S antigen capture and estimation, wells of Immulon-2 microtiter plates (Dynatec Laboratories, Inc., Chantilly, VA) were coated overnight at 4° C with an empirically determined optimal concentration of 1.5 μg of the purified goat IgG anti-protein S in 100 μl pH 9.0, 0.05 M Tris-HCl. Remaining binding sites were blocked with the addition of 200 μl of 2% bovine serum albumin (BSA) in Tris-HCl, pH 7.4 for 2-h at room temperature. Wells were then

washed three times with phosphate buffered saline (PBS) containing 0.002% Tween-20, (Sigma) using an automated plate washer model ELP35 (ADIL Instruments, Strasbourg-Schiltighiem, France).

Purified protein S, used to construct the standard curve (30-0.041 ng/ml), and samples for protein S measurement were serially diluted in 2% BSA/Tris buffer and added in 100 μl volumes to ELISA plate wells.

Following incubation for 2-3-h at room temperature, wells were washed as before and rabbit anti-protein S immunoglobulin (100 μl, 1/2000) diluted in 5% normal goat serum/PBS was added and incubated for 1-h at room temperature. Wells were again washed as above and 100 μl of the HRP-donkey anti-rabbit Ig conjugate diluted 1/1500 in the same buffer was added and incubated for 1-h at room temperature. Wells were washed a final three times and 100 μl of 0.05 M citrate-phosphate buffer, pH 5.8 containing 0.4 mg/ml O-phenylenediamine (Sigma) and 0.0013% hydrogen peroxide, was added to all wells. After 10 min the enzyme-substrate reaction was terminated by the addition of 50 μl 2N H_2SO_4. The change in absorbency at 490 nm was measured in an EL 312e Microplate reader (Bio-tek Instruments, Inc, Winooski, VT). Data reduction and calculation of sample protein S values were completed with the Bio-tek KinetiCal software package. Data presented represent measurement averages (mean ± 1 standard deviation).

Additionally, a commercial protein S ELISA kit (Diagnostica Stago, Asnières, France) was purchased and evaluated for sensitivity of protein S detection and utility in measuring protein S levels in culture fluids.

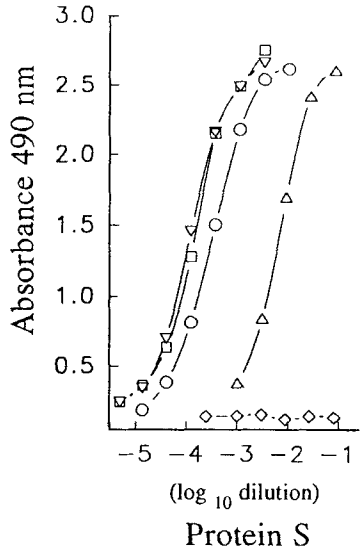

FIGURE 1. Polyclonal antibody capture and detection ELISA for human protein S. The linear-log plot shows parallelism of protein S antigen capture from plasma (▽), serum (□), culture fluid of a human microvascular endothelial cell line (△), and protein S purified from plasma (○). Fetal bovine serum was used as a negative control (◇).

RESULTS

Assay characteristics

1. Standard curve and data analysis

Fig. 1 is a linear-log transformed plot of end-point ELISA data showing the parallelism of protein S antigen capture from serum, plasma, tissue culture fluid and the purified plasma protein S sample. Parallelism is indicative of the absence of sample matrix effects or interfering or competing compounds and comparable binding affinities for protein S in the different samples tested. Data reduction used a linear 4-parameter curve fit program (KinetiCal

software ver. 2.03, Bio-Tek) of standard curve data to determine sample protein S concentrations (not shown). With this analysis, sample protein S values were determined from the linear dynamic range of the standard curve, which was about 250-fold similarly seen in the transformed plots of Fig. 1.

2. Precision

The mean coefficient of correlation (r^2) of the dose response curves for 24 assays averaged 0.997. The mean, standard deviation (SD) and coefficient of variation (CV) for three internal controls for 24 between-assays were 13.91 ± 1.47 (10.6 %), 1.08 ± 0.15 (14.6 %) and 0.210 ± 0.03 (14.2 %) ng/ml, respectively.

3. Accuracy, sensitivity and specificity

Assay accuracy, sensitivity and specificity was demonstrated, based upon observed and expected values, by determining protein S levels in selected biological fluids described below.

Measurement of protein S in different biological samples

1. Tissue culture fluids

Protein S was measured in 24-h culture fluids from confluent monolayers of 6-cell lines of human origin including hepatocytes (HepG-2), microvascular endothelial cells (HMEC-1), brain (U87, H4), bladder (T24) and colon (SW480). Additionally, protein S was measured in 3-primary cultures of human umbilical vein, microvascular and aortic endothelial cell culture fluids. The rate of protein S production in these 9-cell lines ranged from

TABLE 1

ELISA QUANTIFIED PROTEIN S CONCENTRATIONS in 24-HOUR CELL CULTURE FLUIDS

Cell Culture	Origin	Protein S ng/10^5 cells
HAEC	Aortic EC	1.270
HMEC†	Dermal EC	1.163
HUVEC	Umbilical vein EC	0.643
HMEC-1†	Transfected foreskin EC	1.030
HepG-2	Hepatoma	2.129
U87	Glioblastoma	3.066
H4	Neuroglioma	0.768
SW480	Colon	0.850
T24	Bladder	0.210
HL60	Promyelocytic	<0.080
Molt-4	Lymphocytic	<0.080

† Microvascular origin

approximately 0.2-3 ng/10^5 cells/24 hr period. Protein S was not detected in the 24-h or later culture fluids from cell lines HL60 or Molt 4 (Table 1) that were also shown not to produce protein S antigen by Western blot analysis or to express protein S mRNA in Northern blot analysis (26), further confirming the specificity of the assay. Culture fluid protein S levels were observed to increase almost linearly with time with all cell lines other than HL60 and Molt 4. Fig. 2 typically shows ELISA measurement of protein S in the HMEC-1 cell line and HUVEC and microvascular culture fluids over a period of 120-h. With this ELISA, we were able to measure protein S in culture fluids from HMEC-1 confluent monolayers as early as 1-h after a complete change to fresh medium, reflecting a rate of synthesis and release of protein S of 0.086 ± 0.0096 ng/ml of culture fluid or approximately 1077 molecules of

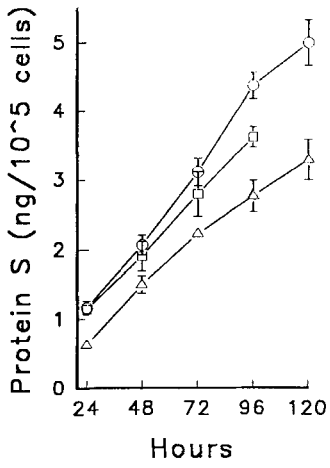

FIGURE 2. ELISA measurements of the accumulation of protein S in cell culture fluids from a transfected microvascular endothelial cell line (HMEC-1) (○), human microvascular (□), and umbilical vein (△) endothelial cell cultures over a 120-hour period.

protein S per cell after 1-h. Fetal bovine serum, at all dilutions, was nonreactive in the assay.

2. Plasma

We further tested the accuracy and specificity of the assay by measurement of protein S in selected plasma samples. The incubation time to effect dissociation of the protein S-C4BP complex in plasma and capture in ELISA was determined empirically. From fig. 3, which shows absorbance as a function of incubation time, it is apparent that plasma diluted 1/40000 requires about 2-h for equilibration and a minimum of 3-h for dilutions ranging from 1/2500-20000. Lower plasma dilutions reportedly require an overnight incubation to effect dissociation of the protein S-C4BP

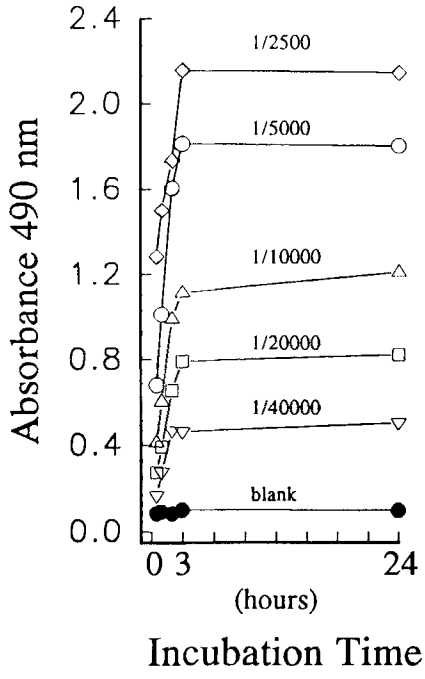

FIGURE 3. ELISA showing the effect of dilution and time on the measure of total protein S in plasma. The early incubation time points tested were 0, 1, 2 and 3 hours. Note that dilutions between 1/2500 - 20000 plateau after 3-hours and after 2-hours at 1/40000 (see text).

complex (2, 11, 27). The assay determined the mean total and free protein S concentrations of plasma from 10 healthy donors, to be 24.8 ± 3.5 and 9.1 ± 1.7 μg/ml, respectively; these determinations were within 10% of previously reported normal mean values of 25.9 μg/ml and 9.8 μg/ml for total and free protein S, respectively (1), thus reflecting the accuracy of the assay. A normal plasma sample depleted of most protein S by immunoaffinity chromatography was determined to have <1% (0.25 μg/ml) of the normal protein S mean

value. This decreased response from a protein S deficient plasma again confirmed the specificity of the assay.

Measurement of protein S using a commercial ELISA Kit

A commercial ELISA was also tested for utility in measuring protein S in some selected culture fluids. The purified protein S calibrator, in place of the manufactures standard serum, was used in this assay to determine the lower limit of detection. An absorbency of about twice the background level was observed with 1.11 ng/ml of the purified protein S preparation. Protein S levels of selected culture fluids measured in the commercial ELISA were within 10% of those measured in our ELISA.

DISCUSSION

An ELISA assay was chosen as the preferred method to measure tissue culture protein S antigen levels because of the relative ease with which the assay is performed, the high precision attainable with due care and the accuracy and high sensitivity from use of specific and high affinity antibodies. However, we were initially surprised by the lack of available protein S ELISA assays. For example, Linscott's Directory of Immunological and Biological Reagents, Seventh Edition, 1992-93 lists only two protein S measurement kits: an electroimmunoassay (EIA) and radio immunoassay kit. And although several investigators have described ELISA assays to measure protein S antigen, such assays were developed from "in-house" or proprietary reagents that are not generally available (1, 19, 27-29). Recently, an international collaborative study

evaluated three commercial methods (two EIA and an ELISA protocol:the European EIA and ELISA (Diagnostiga Stago) were not listed in Linscott's directory) for measuring protein S in plasma (30). This study did not assess the lower limits of protein S detection but rather the performance of the ELISA in detection of protein S antigen levels of clinical significance in plasma i.e., the lower limits of the normal range. However, using the ELISA, a mean of 4 U/dl ± 1.7 of protein S in an immunodepleted plasma or approximately 4% of the normal protein S level was detected and reported from five different laboratories. Since this (30) and previous studies (1, 19, 27-29) focused explicitly on the problems of determining protein S levels in plasma, we evaluated and found the commercial protein S ELISA also useful for measuring low levels of protein S i.e. those encountered in culture fluids. However, the commercial assay resulted in a cost basis of about $2.86/well compared to .07 cents/well for the polyclonal antisera and miscellaneous reagents used here for ELISA development. In a research environment, where hundreds of samples may need to be evaluated on an ongoing basis, the cost of pre-prepared kits can be prohibitive. The ELISA for protein S described here was developed with emphasis on measurement of low levels of protein S in tissue culture fluids. By using selected plasmas and tissue culture samples, the assay was shown to be accurate and antigen specific. The sensitivity, 0.086 ng/ml, was adequate for measuring low levels of protein S in culture fluids, especially in very early culture fluids or cultures that may experience down-regulation of protein S expression in response to various stimuli.

Preliminary studies of the effects of the tumor necrosis factor cytokine on protein S synthesis by large vein and microvascular endothelial cells using the ELISA described here show a downregulation of protein S in endothelial cell cultures (31). These and other studies to determine the mechanism of this action are in progress. The development of ELISAs or other assays using readily available commercial antisera such as those described here will greatly facilitate the in vitro evaluation of events that link together the cytokine network with the coagulation cascade.

‡ Use of trade names is for identification only and does not imply endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

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