EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 28, No 4, 181-186, December 1996

An enzyme immunoassay for pregnancy-specific β-1 glycoprotein (SP1), employing monoclonal antibodies

Jae-Ho Lee[1,4], Seung-Ryul Kim[3] and Hong Keun Chung[2]

1 Department of Biochemistry, Ajou University School of Medicine, Suwon 442-749, Korea
2 Department of Biochemistry and Cancer Research Institute, College of Medicine, Seoul National University, Seoul 110-744, Korea
3 Department of Biochemistry, College of Medicine, Chungbuk National University, Chongju 361-763, Korea
4 To whom correspondence should be addressed.

Accepted 2 December 1996

Abbreviations: SP1, Schwangerschafts-protein 1; HRP, horseradish peroxidase

Abstract

Two-site binding enzyme immunoassay for pregnancy-specific β-1 glycoprotein (SP1) has been developed using two monoclonal antibodies which can recognize all the three forms of SP1 from human sera. This assay system detects a minimal concentration of 1.25 ng/ml, and shows a linear dose-response curve at a range between 1.4 and 45.0 ng/ml. The recovery rates ranged from 90.0% to 113.3%, and intra-assay coefficients of variation were 15.8% and 8.7% for two different samples. When compared with a commercial EIA kit, this assay system had high correlation coefficient (*r* = 0.92) but with somewhat lower values.

Keywords; enzyme immunoassay, monoclonal antibody, pregnancy-specific β-1 glycoprotein (SP1)

Introduction

Human pregnancy-specific β-1 glycoproteins (Schwangerschafts-protein 1, SP1) are a group of closely related placental glycoproteins and members of the immunoglobulin superfamily (Plouzek and Chou, 1991). Originally, they were discovered independently by Tatarinov and Masyukevich (1970) and Bohn (1971) as one of the pregnancy-specific proteins. These proteins can be produced in human intestinal tissue (Shupert and Chan, 1993), T lymphocytes (Wu *et al.*, 1993) and male reproductive tract (Richardson e*t al.*, 1991). However, they are mainly synthesized in the syncytiotrophoblast of placenta (Heikinheimo e*t al.*, 1981) and secreted into the maternal circulation in increasing amounts as gestation progresses (Braunstein e*t al.*, 1980), reaching a maximum of about 0.2 mg/ml in the 34th week of gestation (Bohn, 1972). In males and non-pregnant females, circulating levels of the protein are less than 3 ng/ml (Searle e*t al.*, 1978). The function of the protein is still unknown, although some studies have indicated an immunosuppressive role during pregnancy (Cerni e*t al.*, 1977).

Clinical studies indicate that the measurement of SP1 may be useful for determining complications of early pregnancy such as threatened abortion (Sterzik *et al.*, 1986) and ectopic pregnancy (Seppala e*t al.*, 1980), and as a marker for monitoring the treatment of choriocarcinoma (Searle e*t al.*, 1978) and germ cell tumors (Rosen e*t al.*, 1979; Englund e*t al.*, 1991). In addition, many tumors including breast (Wurz e*t al.*, 1979), colorectal (Haynes e*t al.*, 1985) and lung (Grudzinskas e*t al.*, 1980; Boucher and Yoneda, 1995) cancers have been reported to produce SP1. However, many reports on the correlation of serum SP1 level with various clinical conditions show considerable discrepancies from reports to reports in each cancer. This may partly be due to differences in the immunological reagents and techniques used for the detection of SP1 (Engvall e*t al.*, 1982).

Many studies suggest strongly that SP1 has a heterogeneous nature (Sorensen, 1982; Chan and Qiu, 1988; Kahn *et al.*, 1989), mainly reveals 3 bands (72, 64 and 54 kDa) on SDS electropherogram (Mueller e*t al.*, 1985; Plouzek and Chou, 1991), and has at least 3 different cDNAs (Kahn e*t al.*, 1989). Therefore, when different batches of polyclonal antisera were used, assays would yield variable results. However, monoclonal antibodies are expected to give reproducible results because of their highly specific and uniform nature. In our previous report (Lee e*t al.*, 1991), we reported production of SP1-specific monoclonal antibodies. The development of two-site binding enzyme immunoassay system for SP1 is described in the present paper.

Materials and Methods

Iodogen, Immunopure binding buffer and elution buffer were purchased from Pierce. (Rockford, IL), RPMI, DMEM, incomplete Freund's adjuvant and fetal bovine serum were from Gibco.(Gaithersburg, MD), polystyrene tube from Nunclon. (Denmark), ^{125}I from NEN Research Products. (Boston, MA), CNBr-activated Sepharose 4B from Pharmacia (Uppsala, Sweden), SP1 assay kit from Behring. (Germany), and horsera-

dish peroxidase (Rz=3.2) from Sigma. (St. Louis, MO). All the chemicals used were of analytical grade.

Preparation of monoclonal antibodies

Hybridoma cell lines established in our previous work (Lee *et al.*, 1991) were cultured in DMEM containing 10% fetal bovine serum, 100 mM hypoxanthine, 16 mM thymidine and gentamycin (50 μg/ml). Ascitic fluids were produced by inoculation of cultured hybridoma cells into the peritoneal cavities of incomplete Freund's adjuvant pre-treated Balb/c mouse (Mueller e*t al.*, 1986). Monoclonal antibodies were precipitated from the ascitic fluids by 50%saturation of ammonium sulfate, followed by dialysis against PBS. After clarification of the dialyzed sample by centrifugation, the sample was applied to a protein A-sepharose column previously equilibrated with Immunopure binding buffer (Pierce). Bound antibodies were eluted by using Immunopure elution buffer (Pierce).

Immunoprecipitation

Partially purified SP1 (Lee *et al.*, 1991) was radio-labelled with ^{125}I using Iodogen (Pierce) according to the manufacturer's instruction. Monoclonal antibodies to SP1 were covalently cross-linked to CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's instruction. The radioiodinated SP1 (7.5 x 10^6 cpm/tube) was incubated with irrelevant mouse antibody-linked Sepharose 4B beads at room temperature for 1 h, followed by centrifugation. The same procedure was repeated for the supernatant. Then, the supernatant was incubated with anti-SP1 monoclonal antibody-linked Sepharose 4B beads at room temperature for 12 h. After decanting the supernatant, the precipitated beads were washed with PBS containing 0.05% Tween-20 until the radioactivity of the wash-out solution became negligible. The beads were incubated with SDS-PAGE sample buffer at 90˚C for 3 min, and the supernatants were subjected to SDS-PAGE. The gel was dried and autoradiographed at -70˚C.

Horseradish peroxidase (HRP)-conjugation

The purified antibodies were conjugated with HRP according to the method of Nakane and Kawaoi (1974). Briefly, amino groups on HRP molecules were blocked with 1-fluoro-2,4-dinitrobenzene, and subsequently HRP was oxidized with sodium periodate to formylate its sugar moiety. After the removal of the remaining sodium periodate by dialysis, the monoclonal antibody solution was added to HRP-aldehyde solution. The conjugate was separated from free HRP by gel filtration on a Sephadex G-200 column. Fractions were collected and their absorbances were measured at 280 nm for protein and 405 nm for HRP heme group.

Preparation of SP1 standards and "zero control serum"

SP1 standards in Enzygnost™ kit (Behring, Germany) were used for this study. Zero control serum was prepared by diluting the sera which contain less than 0.3 ng/ml SP1, when measured by Enzygnost™ EIA kit, to 1:5 with PBS containing 1% BSA.

Two-site binding enzyme immunoassay

Polystyrene tubes were used instead of ELISA plate. The tubes were coated with a monoclonal antibody (SP106-1-12) in 50 mM sodium bicarbonate buffer, pH 9.6 (15 μg/ml) by incubating at 4˚C overnight. After blocking remaining sites with 1% (w/v) bovine serum albumin, SP1 standards or samples (200 μl) were added to each tube. After incubation at 37˚C for 2 h, the tubes were washed three times with distilled water. Then, 200 μl of HRP-labelled antibody solution(1.5 μg/ml SP119-4-1 antibody in PBS containing 1% BSA) was added to each tube. Incubation was carried out at 37˚C for 2 h, and the tubes were then washed three times with distilled water. Subsequently, 200 μl of the substrate solution (100 mM citrate buffer, pH 5.0, containing 2 mg/ml *o*-phenylene diamine and 0.005% H_2O_2) was added, the tubes were incubated at room temperature for 25 min, and then 400 μl of 0.5 N H_2SO_4 solution was added to each tube to stop the enzyme reaction. Absorbance of the solution was measured at 492 nm.

Results

Of the eight monoclonal antibodies against SP1 which we produced (Table 1), SP106-1-12 was selected as a capture antibody and SP119-4-1 as a labelled antibody, because this combination showed relatively high sensitivity with low non-specific binding (data not shown) for measuring three isoforms of SP1 from pregnant serum (Figure 1). The molecular mass of the three isoforms were 68, 64 and 56 kDa, respectively.

A typical dose-response curve is shown in Figure 2. It shows a linear dose-response relationship at the concentration range of 1.4-45.0 ng/ml. The "high-dose hook effect" was observed in the concentrations above 10 μg/ml (Figure 3). However, up to the level of 200 μg/ml, the absorbance of the samples was much higher than that of the sample containing 45.0 ng/ml SP1, which was the highest concentration of the standards.

As shown in Table 2, the lower limit of detection was estimated to be 1.25 ng/ml. The lower limit of detection was defined as that concentration of SP1 which this assay system can show statistically significant difference from the "zero control serum".

Intra-assay variation is assessed by measuring two serum samples with different amounts of SP1. As

Table 1. Isotypes of monoclonal antibody

Antibody	Isotype
125-27-1	IgG1 (κ^a)
106-1-12	IgG1 (κ)
147-5-8	IgG1 (κ)
8-1-7	IgG2a (κ)
119-4-1	IgG1 (κ)
233-4-3	IgG1 (κ)
14-2-6	IgG1 (κ)
225-4-1	IgG1 (κ)

[a] Denotes the isotype of the light chain

Table 2. The lower limit of detection

	Zero control serum	Sample (1.25 ng/ml)
Number of Replicates	27	24
Mean Absorbance at 492 nm	0.052[a]	0.076
Standard Deviation	0.016	0.012

[a] The mean absorbance in "zero control serum" was significantly lower than that in 1.25 ng/ml serum (Student *t*-test, $p < 0.05$).

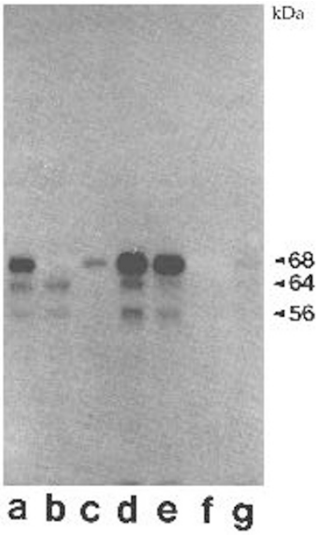

Figure 1. Immunoprecipitation analysis of anti-SP1 antibodies. Partially purified SP1 was radiolabelled with ^{125}I, and subsequently precipitated with Sepharose 4B beads conjugated with monoclonal antibodies, SP119-4-1 (a), SP8-1-7 (b), SP147-5-8 (c), SP106-1-12 (d), and SP125-27-1 (e), normal mouse immunoglobulin (f), and polyclonal anti-SP1 antibody from Enzygnost™ kit (g). The three isoforms of SP1 have the molecular weights of 68, 64 and 56 kDa, respectively.

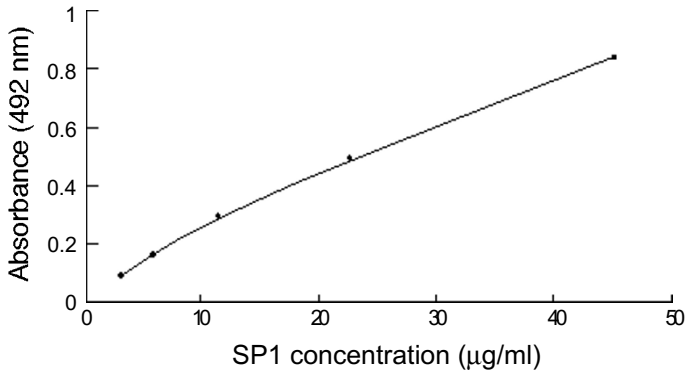

Figure 2. A typical dose-response curve. Polystyrene tubes were coated with monoclonal antibody, SP106-1-12, and the standard samples were added to the tubes. After the initial incubation, tubes were washed with distilled water and subsequently were incubated with HRP-labelled SP119-4-1 antibody. After incubation for 2 h, the tubes were washed with distilled water and subsequently incubated with substrate solution containing *o*-phenylene diamine. Absorbance of the solution was measured at 492 nm.

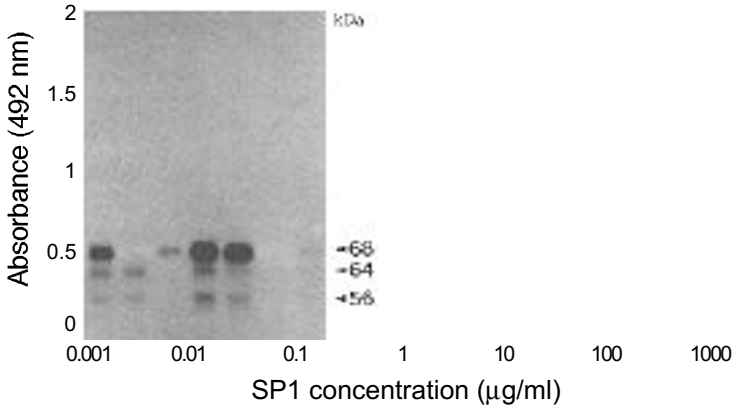

Figure 3. High dose "hook effect". The serum containing 200 μg/ml SP1 was serially diluted, and the absorbance was measured using our two-site binding EIA system.

shown in Table 3, the intra-assay coefficients of variation were 15.8% for sample 1 (1.25 ng/ml) and 8.7% for sample 2 (14.4 ng/ml). The recovery of the assay was assessed by adding aliquots of serum with a high SP1 concentration to a serum with a low SP1 concentration. The SP1 concentrations were measured and compared with estimated concentrations. The recoveries ranged from 90.0% to 113.3% (Table 4). When compared with the Enzygnost™ EIA kit, this assay system indicated high correlation coefficient (r = 0.92) with somewhat lower values than the commercial kit (Figure 4).

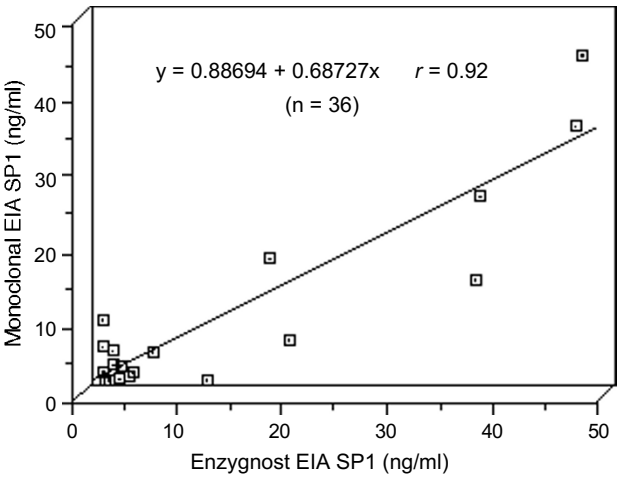

Figure 4. Comparison of SP1 concentrations measured by our monoclonal EIA system with the Enzygnost™ EIA kit. Sera from 36 normal human subjects were used to measure the values of SP1 using our EIA system and Enzygnost™ EIA kit.

Table 3. Intra-assay variation.

	Sample 1	Sample 2
Concentration (ng/ml)	1.25	14.4
Numbers of Replicates	24	19
Mean Absorbance at 492 nm	0.076	0.311
Standard Deviation	0.012	0.027
Coefficient of Variation	15.8%	8.7%

Table 4. Recovery of SP1.

SP1 added (ng/ml)	value obtained (ng/ml)	obtained value - endogenous value[a]	SP1 recovery (%)
1.0	2.9	0.9	90.0
3.0	5.4	3.4	113.3
10.0	12.0	10.0	100.0
30.0	31.6	29.6	98.7

[a] endogenous value = 2.0 ng/ml

Discussion

Of the eight monoclonal antibodies produced against SP1, three antibodies were shown by immunoprecipitation reaction to recognize all the three different isoforms of SP1 in serum (Figure 1). In order to measure all forms of SP1, these three antibodies were chosen in the present assay system. Every possible combinations employing three antibodies as capture antibodies or labelled antibodies were tried. However, the assay system using SP106-1-12 as a capture antibody and SP119-4-1 as a labelled antibody was chosen, because this combination showed relatively high sensitivity with low non-specific binding for measuring SP1.

A typical standard curve shows that our assay system has a linear dose-response relationship in the concentration range between 1.4 and 45 ng/ml. It is comparable with Enzygnost™ EIA system (3 to 300 ng/ml). In immunoradiometric assays, a paradoxical reversal of the dose-response relation, so called “hook effect”, often emerges at a high concentration range (Janina e*t al.*, 1979). This can sometimes be eliminated by using sequential rather than simultaneous application of different antibodies (Chard, 1987). Although two antibodies were used sequentially in this study, “hook effect” was still observed above the concentration of 10 μg/ml. However, even when we measure serum samples with as high as about 200 μg/ml SP1, the signal still remains far higher than that of the sample containing 45 ng/ml SP1, which is the highest concentration among the standards. Therefore, practically none of the samples within the range of “hook effect” can be underestimated, since investigators will always dilute them to measure correct amount of SP1.

The lower limit of detection of this assay method was 1.25 ng/ml. This value is much higher than the lower

limit of detection by Enzygnost™ EIA kit (Behring), but is still lower than the one reported in a previous study using their monoclonal antibodies (Engvall *et al*., 1982). Also, the cut-off value for deciding pregnancy is 2 (Pak *et al*., 1988) or 3 ng/ml (insert manual of Enzygnost™ EIA kit) which are higher than the lower limit of detection of our assay system.

The intraassay coefficients of variation of our system were 15.8% for sample 1 (1.25 ng/ml) and 8.7% for sample 2 (14.4 ng/ml). These values were somewhat higher than those reported by Alexander (1981) (2.6%) and Macdonald *et al*. (1979) (6.5, 7.4%). When compared with Enzygnost™ EIA kit, our assay system showed a high correlation coefficient, but has a tendency to have lower value. This may be due to the differences in reactivity of polyclonal and monoclonal antibodies toward different forms of SP1.

In conclusion, it is not certain which system is better in terms of accuracy. However the assay system using monoclonal antibodies should be more consistent in assay than those using polyclonal antibodies, because they do not have batch variations.

Acknowledgements

We thank Professor W. K. Paik, Department of Biochemistry, Ajou University School of Medicine, for critical review of this manuscript and Dr. In Kyoung Lim, Department of Biochemistry, Ajou University School of Medicine, for her generous permission to use some facilities and materials used in this study.

References

Alexander, L. C. (1981) Enzyme-linked immunoassay compared with electroimmunoassay for routine estimation of pregnancy-specific glycoprotein (SP1) in sera during pregnancy. *Med. Lab. Sci.* 38: 77-83

Bohn, H. (1971) Nachweis und Charakterisierung von Schwan-gerschaft-Proteinen in der menschlichen Plazenta, sowie ihre quantitative immunologische Bestimmung im Serum schwangerer Frauen. *Arch. Gynecol.* 210: 440-457

Bohn, H. (1972) Isolierung und Characterisierung des Schwan-gerschaftsspezifischen-glykoproteins. *Blut* 24: 292-302

Boucher, L. D. and Yoneda, K. (1995) The expression of trophoblastic cell markers by lung carcinomas. *Hum. Pathol*. 26: 1201-1206

Braunstein, G. D., Rasor, J. L., Engvall, E. and Wade, M. E. (1980) Interrelationships of human chorionic gonadotrophin, human placental lactogen, and pregnancy-specific 1-glycoprotein throughout normal human gestation. *Am. J. Obstet. Gynecol.* 138: 1205-1213

Cerni, C., Tatra, G. and Bohn, H. (1977) Immunosuppression by human placental lactogen (HPL) and the pregnancy-specific 1-glycoprotein (SP1). *Arch. Gynaekol.* 223: 1-6

Chan, W. W. and Qiu, W. R. (1988) Human pregnancy-specific glycoprotein is encoded by multiple genes localized on two chromosomes. *Am. J. Hum. Genet.* 43: 152-159

Chard, T. (1987) *An introduction to radioimmunoassay and related techniques*. 3rd Ed., p. 136, Elsevier, New York

Englund, A. T., Geffner, M. E., Nagel, R. A., Lippe, B. M. and Braunstein, G. D. (1991) Pediatric germ cell and human chorionic gonadotrophin-producing tumors. Clinical and laboratory features. *Am. J. Dis. Child*. 145: 1294-1297

Engvall, E., Masato, M. and Erkki, R. (1982) Monoclonal antibodies in analysis of oncoplacental protein SP1 *in vivo* and *in vitro*. *Cancer Res.* 42: 2028-2033

Grudzinskas, J. S., Coombes, R. C., Ratcliffe, J. G., Gordon, Y. B., Powles, T. J., Munro, N. A. and Chard, T. (1977) Specific and sensitive determination of pregnancy-specific glycoprotein by radioimmunoassay. A new pregnancy test. *Lancet* 1: 333-335

Haynes, W. D. G., Shertock, K. L., Skinner, J. M. and Whitehead, R. (1985) The ultrastructural immunohistochemistry of oncofetal antigens in large bowel carcinomas. *Virchows Archiv* 405: 263-275

Heikinheimo, M., Saksela, O., Lehtovirta, P., Seppala, M. and Bohn, H. (1981) Cultured human syncytiotrophoblasts synthesize pregnancy-specific beta-1-glycoprotein (SP1). *Acta Pathol. Microbiol. Scand. Sect. C* 89: 139-144

Janina, K., Ida, C. and Saul, W. R. (1979) Radioimmunoassay of "pregnancy-specific" glycoprotein (SP1). *Clin. Chem.* 25: 577-580

Kahn, W. N., Osterman, A. and Hammastrom, S. (1989) Molecular cloning and expression of cDNA for a carcinoembryonic antigen-related fetal liver glycoprotein. *Proc. Natl. Acad. Sci. U.S.A.* 86: 3332-3336

Lee, J. H., Chung, J. H. and Chung, H. K. (1991) Purification of pregnancy-specific beta-1 glycoprotein from human placenta and production of its monoclonal antibody. *Mol. Cells* 1: 235-239

Macdonald, D. J., Belfield, A., Steele, C. J., Mack, D. S. and Shah, M. M. (1979) The quantitation of pregnancy specific beta 1-glycoprotein by enzyme linked immunoassay. *Clin. Chim. Acta* 94: 41-49

Mueller, U. W., Hawes, C. S. and Jones, W. R. (1986) Monoclonal antibody production by hybridoma growth in Freund's adjuvant primed mice. *J. Immunol. Methods* 87: 193-196

Nakane, P. and Kawaoi, A. (1974) Peroxidase-labelled antibody. A new method of conjugation. *J. Histochem. Cytochem.* 22: 1084-1091

Pak, C. H., Steven, Y. W. and Grace, W. K. (1988) Diagnosis of early pregnancy by enzyme immunoassay of Schwangerschafts-protein 1. *Fertil. Steril*. 49: 76-80

Plouzek, C. A. and Chou, J. Y. (1991) Isolation and characterization of a human amnion epithelial cell line that expresses the pregnancy-specific beta 1-glycoprotein gene. 129: 950-958

Richardson, L. L., Chan, W. Y. and Dym, M. (1991) Localization of pregnancy-specific beta 1-glycoprotein in the male reproductive tract of the rat by in situ hybridization. *Biol. Reprod.* 45: 704-710

Rosen, S. W., Kaminska, J., Calvert, I. S. and Aaronson, S. A. (1980) Human fibroblasts produce "pregnancy-specific" beta-1 glycoprotein *in vitro*. *Am. J. Obstet. Gynecol*. 137: 525-529

Searle, F., Leake, B. A., Bagshawe, K. D. and Dent, J. (1978) Serum SP1-pregnancy specific β1 glycoprotein- in choriocarcinoma and other neoplastic diseases. *Lancet* I: 579-580

Seppala, M., Venesmaa and Rutanen, E. M. (1980) Pregnancy-specific beta-1 glycoprotein in ectopic pregnancy. *Am. J. Obstet. Gynecol.* 136: 189-193

Shupert, W. L. and Chan, W. Y. (1993) Pregnancy specific beta 1-glycoprotein in human intestine. *Mol. Cell. Biochem.* 120: 159-170

Sorensen, S. (1982) Heterogeneity of the pregnancy-specific 1-glycoprotein (SP1). *Clin. Chim. Acta* 121: 199-208

Sterzik, K., Wenske, C., Rossmanith, W. and Benz, R. (1986) Beta-1-glycoprotein determination in normal and disturbed pregnancy. *Int. J. Gynaecol. Obstet.* 24: 65-68

Tatarinov, Y. S. and Masyukevich, V. N. (1970) Immunochemical identification of new

beta-1 globulin in the blood serum of pregnant women. *Byull. Eksp. Biol. Med.* 69: 66-68

Wu, S. M., Bazar, L. S., Cohn, M. L., Cahill, R. A. and Chan, W. Y. (1993) Expression of pregnancy-specific beta 1-glycoprotein genes in hematopoietic cells. *Mol. Cell. Biochem.* 122: 147-158

Wurz, H. (1979) Serum concentrations of SP1 (pregnancy-specific β1-glycoprotein) in healthy, nonpregnant individuals, and in patients with nontrophoblastic malignant neoplasms. *Arch. Gynecol.* 227: 1-6